Immunopathogenesis of chronic *Mycobacterium marinum* infection in adult zebrafish (*Danio rerio*)

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Doctor of Philosophy

UNIVERSITY OF STIRLING

By

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DECLARATION

I hereby declare that the work and results presented in this thesis was conducted by me at the Institute of Aquaculture, University of Stirling, Scotland.

The work presented in this thesis has not been previously submitted for any other degree or qualification.

The literature consulted has been cited and where appropriated, collaborative assistance has been acknowledged,

Stirling, March 2014

Gilta Jaeckel

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I would like to thank my PhD advisors, Professor Alexandra Adams, Dr Kim D. Thompson and Professor James E. Bron for supporting me during my last years the guidance. Without their guidance, patients and knowledge this achievement would not have been possible.

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ABSTRACT

Tuberculosis (TB) is still a global epidemic disease despite its discovery over 100 years ago. It is caused by *Mycobacterium tuberculosis*, which invades and replicates within macrophages, key cells of the innate immune system. The hallmark of tuberculosis is the granuloma which is an accumulation of *Mycobacterium*-infected cells surrounded by immune cells, and the containment of the bacteria is assured as long as the host immune response remains intact. Despite a well-developed immune response in the infected host, reactivation of latent tuberculosis infection (LTBI) may occur through the introduction of other bacterial pathogens, re-infection with *M. tuberculosis* or due to other immunosuppression, e.g. AIDS or cancer.

The zebrafish–*M. marinum* model provides an ideal system for examining the pathogenesis of tuberculosis and the associated immune response of the host due to its vertebrate-like immune system, and the close phylogenetic relationship of M. marinum to *M. tuberculosis*. Granuloma formation and immune response to *M. marinum* have been investigated mainly in zebrafish embryos or larvae, which lack an adaptive immune response, and little work has been performed in adult fish. This complicates the transfer of findings in these models to chronic, latent or re-activated disease stages in humans, where adaptive immunity plays an important part.

The aim of the research presented here was to investigate the immune response of the adult zebrafish to *M. marinum* infection, with the focus on the kidney as one of the major immune organs in fish. The results obtained support further use of the adult zebrafish-*M. marinum* model for human tuberculosis infections in the future.

In the present study, adult zebrafish were infected with low doses of *M. marinum* (NCIMB 1297 or NCIMB 1298) and the kidney was investigated for histopathological changes in the form of granulomas over a period of two months (Chapter 3). No granulomas were detected in the fish infected with *M. marinum* NCIMB 1298 while in zebrafish infected with NCIMB 1297, macrophage aggregation and

granuloma formation were detected as early as day 11 post-infection. Occurrence and severity of granulomas and the presence of replicating bacteria increased over time, resulting in a high density of non-caseating and caseating granulomas in the head and posterior kidney after two months of infection.

Interleukin 1 beta (IL-1β), Interleukin-12 (IL-12), Tumor necrosis factor alpha (TNFα) and Interferon gamma (IFNγ) have been shown to be important cytokines functioning in defence against tuberculosis, especially IFNγ which is considered to play an important part in acute, chronic and latent tuberculosis. Changes in gene expression of these immune genes in adult zebrafish were investigated over the first two weeks of infection with *M. marinum* NCIMB 1298 and NCIMB 1297. The results obtained in the first week after infection were inconclusive for both strains investigated. In agreement with the results presented in Chapter 3, no specific immune response was detectable in fish infected with *M. marinum* NCIMB 1298. However, after 14 days, a high-fold change in IL-12 and TNFα expression were detected in fish infected with *M. marinum* NCIMB 1297, while IL-1β showed no changes compared to the control fish. Furthermore, no IFNγ expression was detectable over the first two weeks of infection. The delay in the expression of IL-12 and the lack of IFNγ expression can be explained by the ability of *M. marinum* to manipulate the host immune response, as described for *M. tuberculosis* and other intracellular bacteria.

Besides *in vivo* investigations of the host-pathogen interactions, *in vitro* primary macrophage cultures from individual zebrafish kidneys were developed to investigate macrophage-specific gene expression to *M. marinum* infection (Chapter 4). Although the results looked promising, further optimization is required before the results of the *in vitro* assays can be fully compared to the *in vivo* results.

Our understanding of reactivation in latent tuberculosis infection (LTBI) both in healthy and immune compromised individuals is insufficient and is delaying the development of treatments for the disease. Therefore, the transcriptome profile of long-term infections (26 weeks) with *M. marinum* NCIMB 1297 in adult zebrafish was

investigated to determine whether the gene expression in this model is comparable to LTBI in humans or other vertebrate model organisms (Chapter 5). In addition, transcriptome profiling was investigated in a group of long-term infected zebrafish exposed to stress to induce re-activation of the disease. Expression profiles in the long-term infected fish and the infected plus stressed fish differed from each other and displayed similar gene profiles to those found in the latent or re-activated disease states, respectively, in human and other vertebrate models. Infected fish displayed a profile highlighted by IFNγ, TNFα, NOS2b and IL-8 expression alongside activating and regulatory T cell responses, including involvement of cytotoxic T cells (CTLs). The transcriptome profile of the group of fish that had been infected and then stressed was distinguished by the lack of IFNγ expression and reduction in TNFα and NOS2b expression, as well as a lack of T cell response compared to the infected fish.

In conclusion, the results obtained from Chapters 3 and 4 showed that *M. marinum* NCIMB 1298 is non-pathogenic to zebrafish. Infection with *M. marinum* NCIMB 1297, on the other hand, resulted in a similar immune response to that described for human and other mammalian vertebrate models (Chapters 3-5). These results support the use of the adult zebrafish-*M. marinum* model to investigate LTBI and disease reactivation, and will aid our understanding host-pathogen interactions for tuberculosis in the future.

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Talks:

Jaeckel, G., J. E. Bron, K. D. Thompson, J. Lamb, A. Adams (2012). Innate immune response and granuloma formation in the kidney of adult zebrafish (*Danio rerio*) challenged with *Mycobacterium marinum,* Institute of Aquaculture, Lunch time Seminar, 26.09.2012

Poster:

Jaeckel, G., J. E. Bron, K. D. Thompson, J. Lamb, A. Adams (2013). Innate pro-inflammatory immune response and granuloma formation in the kidney of adult zebrafish (*Danio rerio*), First International Conference of Fish and Shellfish Immunology, 25-28 June 2013, Vigo, Spain.

Jaeckel, G., J. E. Bron, K. D. Thompson, J. Lamb, A. Adams (2012). Innate immune response and granuloma formation in the kidney of adult zebrafish (*Danio rerio*) challenged with *Mycobacterium marinum*, 3nd Institute of Aquaculture PhD Research Conference, 24th October 2012, Stirling, United Kingdom.

Jaeckel, G., J. E. Bron, K. D. Thompson, J. Lamb, A. Adams (2011). *In vivo* and *in vitro* gene expression of key cytokines isolated from kidney and coelomic macrophages of zebrafish (*Danio rerio*) infected with *Mycobacterium marinum*, 15th EAFP International Conference on Diseases of Fish and Shellfish, 11th – 15th September 2011, Split, Croatia

Jaeckel, G., J. E. Bron, K. D. Thompson, J. Lamb, A. Adams (2010). Development of zebrafish (*Danio rerio*) as a model to study the reactivation of latent tuberculosis infection (LTBI), 2nd Institute of Aquaculture PhD Research Conference, 28th June 2010, Stirling, United Kingdom

LIST OF ABBREVIATIONS

°C	degree Celsius
µg	microgram
µL	microliter
µm	micrometre
µM	micro molar
ABCB3l1	ATP-binding cassette, sub-family B (MDR/TAP), member 3 like 1
AIDS	Acquired Immunodeficiency Syndrome
AKT2l	v-akt murine thymoma viral oncogene homolog 2, like
ANOVA	analysis of variance
APC	antigen presenting cell
ARALAM	arabinofuranosyl-terminated LAM
ARF	aquatic research facility
aRNA	amplified RNA
ATCC	American Type Culture Collection
B2M	beta-2-microglobulin
BCG	Bacillus Calmette–Guérin
BCP	Bromochloropropane
BLAST	basic alignment search tool
bp	base pairs
BSL	biosafety level
C3	complement component 3
C3a	complement component 3a
C3c	complement factor c3c
C6	complement factor 6
CALRl	calreticulin-like
CAPN1a	calpain 1
CARD9	caspase recruitment domain-containing protein 9
CC3c3	complement component c3c
CCL8	C-C-motif chemokine 8
CCL-C20c	C-C-motif chemokine C20c
CD	cluster of differentiation
cDNA	complementary DNA
CFB	complement component B
CFU	colony forming units
cm	centimetre
CR	complement receptor
cRNA	*in vitro* copied RNA
CRP	c reactive protein
Ct	threshold cycle
CTLs	cytotoxic T cells
CTSl1a	cathepsin L 1 a
Cy3/ Cy5	cyanine 3/ cyanine 5
CXCL-c1c	chemokine (C-X-C motif) ligand c1c
CXCL 11l	chemokine (C-X-C motif) ligand 11 like
CxCl12a	chemokine (C-X-C motif) ligand 12a
CXCL14	chemokine (C-X-C motif) ligand 14
CYCSb	cytochrome c, somatic b
CYP2AA1	cytochrome P450, family 2, subfamily AA, polypeptide 1
CYP2P6	cytochrome P450, family 2, subfamily P, polypeptide 6
CYP2P7	cytochrome P450, family 2, subfamily P, polypeptide 7
d.p.i	days post infection

dH_2O	distilled water
DNA	deoxyribonucleic acid
DRAM1	DNA-damage regulated autophagy modulator 1
DUSP2	cual specificity protein phosphatase 2
e.g.	example
Elong1α	translation elongation factor 1 α
EMBL-EBI	European Molecular Biology Laboratory - European Bioinformatics Institute
et al.	*et alia* (and others)
EtBr	ethidium bromide
F3b	coagulation factor IIIb
F5	coagulation factor V
FASLG	Fas ligand (TNF superfamily, member 6)
Fig	figure
FN1	fibronetin 1
g	gram
GADD45AA	growth arrest and DNA-damage-inducible, alpha, a
GADD45Ab	growth arrest and DNA-damage-inducible, alpha, b
GALT	gut-associated lymphoid tissue
GAPDH	glycerinaldehyde-3-phosphat-dehydrogenase
GFP	green fluorescent protein
GP1bb	glycoprotein Ib
h	hours
h.p.i.	hours post-infection
H&E	haematoxylin and eosin
HBSS	Hank's buffered salt solution
HIV	Human Immunodeficiency Virus
HRASA	v-Ha-ras Harvey rat sarcoma viral oncogene homolog a
i.e.	*id est* (that is)
IFNγ	interferon gamma
Ig	immunoglobulin
IGRA	Interferon gamma release assay
IHNV	Infectious hematopoietic necrosis virus
IL	interleukin
IL-15a	IL-15 receptor alpha chain
I.P.	Intraperitoneal
IPNV	Infectious pancreatic necrosis virus
IRAK3	interleukin-1 receptor-associated kinase 3
IRF	interferon regulatory factors
ISKNV	Infectious spleen and kidney necrosis virus
ISLR2	immunoglobulin superfamily containing leucine-rich repeat 2
ITGA2b	integrin alpha 2b
KAAS	KEGG Automatic Annotaion Server
kDa	kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	litre
L-15	Leibovitz-15
LAM	lipoarabinomannan
LM	Lipomannan
LPA	linear polyacrylamide
LPS	lipopolysaccharide
LTBI	latent tuberculosis infection
M	molar
MANLAM	mannosylated Lipoarabinomannan
MAP	mitogen-activated protein

MAP3K8	MAP kinase kinase kinase 8
MAPK	MAP kinase
MAPKAPK3	MAP kinase-activated protein kinase 3
MBL	mannan-bindin lectin
MCP	monocyte chemotactic protein
MDR	multidrug resistant
MOB1Bb	MOB kinase activator 1Bb
m.p.i.	months post infection
mg	milligram
MHC	major histocompatibility class
min	minute
mL	millilitre
mM	millimolar
MMP	matrix metalloproteinase
MR	mannose receptor
mRNA	messenger ribonucleic acid
mta2	metastasis associated 1 family member 2
MTB	*Mycobacterium tuberculosis*
NBF	neutral buffered formalin
NCBI	National Center for Biotechnology Information
NCF4	neutrophil cytosol factor 4
NCIMB	National Collection of Industrial Food and Marine Bacteria (UK)
NF-KB	nuclear factor kappa-light-chain-enhancer
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
NFKBIA	NF-κB Inhibitor Alpha
NFKBIAA	NFKBIA alpha
NFKBIAB	NFKBIA beta
ng	nanogram
NK cell	natural killer cell
NLR	NOD-like receptor
nm	nanometre
NNV	Nervous Necrosis Virus
NOS2	nitric oxide synthase
OPTN	optineurin
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PEBP1	phosphatidylethanolamine binding protein 1
PIM	Phosphatidylinositol Mannoside
PILAM	phosphoinositol-capped LAM
PRKCq	protein kinase C, theta
PPD	purified protein derivative
PROC	protein C
PRR	pattern recognition receptor
PSME1	proteasome activator subunit 1
PTGIS	prostaglandin I2 synthase
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RD	region of difference
RHOAE	rhoae ras homolog gene family, member Ae
RIPK2	receptor-interacting serine-threonine kinase 2
RLR	RIG-like receptor
RNA	ribonucleic acid

RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RPL13a	ribosomal protein L13a
rRNA	ribosomal RNA
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SAA	serum amyloid A
SD	standard deviation
SE	standard error
sec	seconds
SESN1	sestrin 1
SERPIND1	serine proteinase inhibitor, clade D, member 1
ST6GAL1	beta-galactoside alpha-2,6-sialyltransferase 1
SVCV	Spring viraemia of carp virus
TAE	Tris acetate EDTA
TAPA1	CD81 antigen
TAPSIN	TAP binding protein
TB	tuberculosis
Tc	T-cytotoxic cells
TGFb	transforming growth factor beta
TGFb3	transforming growth factor beta 3
Th	T-helper cells
TLR	toll like receptors
TNF	tumor necrosis factor
TNFα	tumor necrosis factor alpha
TNFAIP2	TNFα induced protein 2
TNFAIP3	TNFAIP3-interacting protein 1-like
TNF b	tumor necrosis factor beta
TNFRSF9a	tumor necrosis factor receptor superfamily, member 9a
TNFRSF13b	tumor necrosis factor receptor superfamily, member 13b
TRIM35-9	tripartite motif containing 35-9
SOCS1	suppressor of cytokine signaling 1
TST	Tubercle skin test
TXN	thioredoxin
UK	United Kingdom
USA	United States of America
UV	ultraviolet
v/v	volume/volume
VHSV	viral haemorrhagic septicaemia virus
WHO	World Health Organisation
w/v	weight/volume
XDR-TB	extensively drug-resistant tuberculosis

1 Literature Review

1.1 *Tuberculosis*

1.1.1 Historical Background

Tuberculosis is one the oldest diseases in human history and has been given many names over the centuries. Ancestors of the modern *Mycobacterium tuberculosis* strain were present in Africa about 3 million years ago (Gutierrez *et al.* 2005; Daniel 2006). The most commonly accepted hypothesis of how humans first became infected with mycobacteria is related with the beginning of urban settlements and the domestication of aurochs such as cattle about 8000 years ago (Gomez i Prat and de Souza 2003; Daniel 2006). The consumption of meat and milk, which was most likely contaminated with mycobacteria, is considered to be the original source of introduction into mankind (Rothschild *et al.* 2001; Daniel 2006). Evidence of tuberculosis in early human history can be found all over the world. Mycobacterial DNA was detected in Egyptian and Peruvian mummies (Salo *et al.* 1994; Nerlich *et al.* 1997; Konomi *et al.* 2002; Zink *et al.* 2003; Daniel 2006) supporting the idea that mycobacteria crossed the Bering strait during human migration about 33000 years ago (Daniel 2006). Other evidences of the existence of tuberculosis in ancient human history can be found in original texts from India and on pottery from China about 3000 – 2000 years ago (Daniel 2006). Recent analysis indicates that *M. tuberculosis* was introduced to modern humans over 70000 years ago (Comas *et al.* 2013).

The most important discovery related to tuberculosis was made in 1882 by Robert Koch, who used a specific staining method, the Ziehl-Neelsen stain, to visualize *M. tuberculosis*, which he used to discover the etiology of tuberculosis. Some years after that Koch discovered tuberculin (PPD) as a diagnostic tool (Herzog 1998; Kaufmann and Schaible 2005; Daniel 2006).

Although it has been over a century since the discovery of *M. tuberculosis*, the disease is still one of the main bacterial causes of death (Young *et al.* 2009; WHO 2013). The natural history of tuberculosis depends on the host's immunity, hypersensitivity and factors such as nutritional status, associated disease and vaccination. Tuberculosis is a treatable disease, but despite this, it remains a major problem in under-developed countries with high rates of new infections in Africa and Asia (Fig. 1-1). The World Health Organisation (WHO) reported 8.6 million new cases of tuberculosis in 2012. About 13% of those infected are human immunodeficiency virus (HIV) positive and tuberculosis is the major cause of death in patients with AIDS/ HIV (Fox and Menzies 2013; WHO 2013).

Increasing proportions of these new infections are caused by multidrug-resistant (MDR) or extensively drug-resistant mycobacteria (XDR-TB) (Young *et al.* 2009; WHO 2013) and there is a worrying trend towards an increasing number of drug-resistant strains worldwide (Lakhani *et al.* 2010; Zignol *et al.* 2012; WHO 2013). The number of countries that have reported at least one drug-resistant strain (Fig. 1-2) is increasing annually.

Recently, new drug resistant strains of *M. tuberculosis* have been discovered in India and Southern Brazil (Gandhi *et al.* 2010; Atre *et al.* 2011; Teotonio *et al.* 2011; Matteelli *et al.* 2012; Perizzolo *et al.* 2012; WHO 2013). The exact number of drug-resistant strains is still unknown due to unreported cases from most African countries (Fig. 1-3).

As a result of this new alarming trend of drug-resistant bacilli, the urge for controlling tuberculosis by a deeper understanding of this disease is more important than ever.

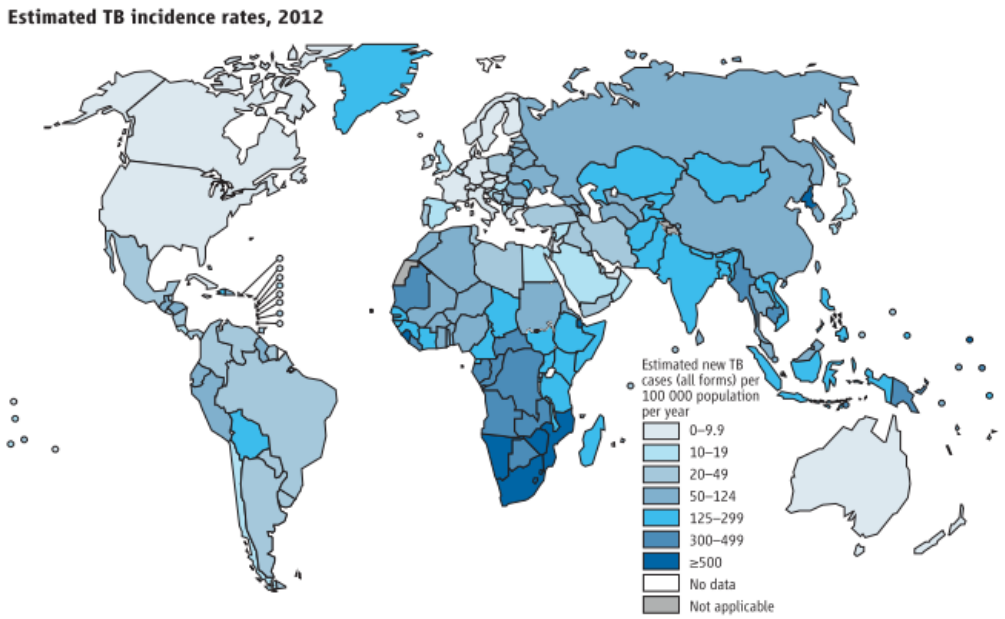

Figure 1-1: Estimated TB incidence rates in 2012 calculated by the WHO. Map shows the number of new TB cases per 100000 populations illustrated in different blue shades. Data was collected and illustrated in 2012 by the WHO in their annual TB control report (WHO 2013).

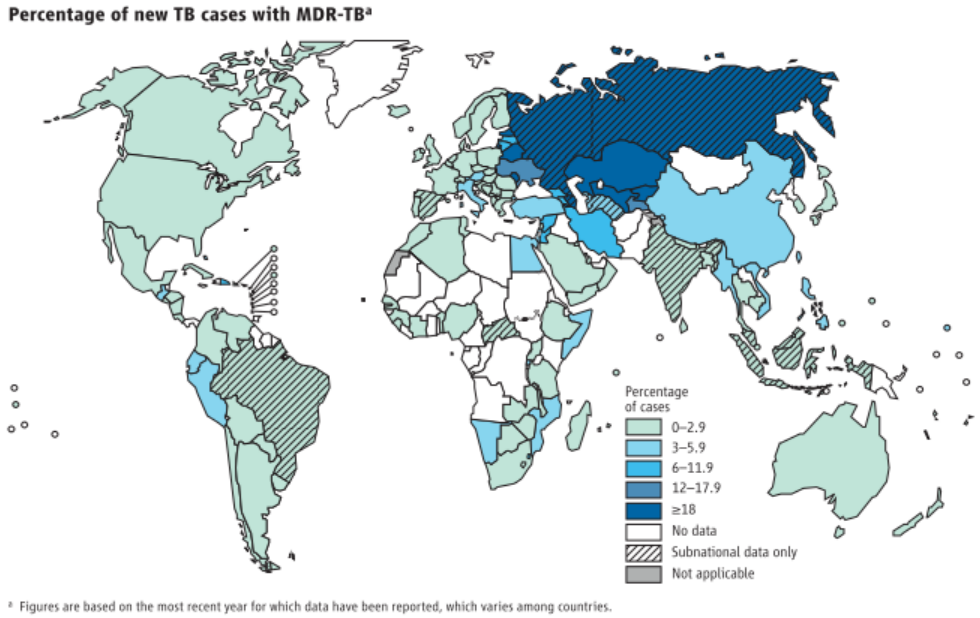

Figure 1-2: Distribution of countries and territories reporting at least one case of MDR-TB. The map highlights the countries (blue) with at least one reported case of drug resistant *M. tuberculosis* strain. Data was collected and illustrated in 2013 by the WHO in their annual multidrug and extensively drug-resistant TB report (WHO 2013).

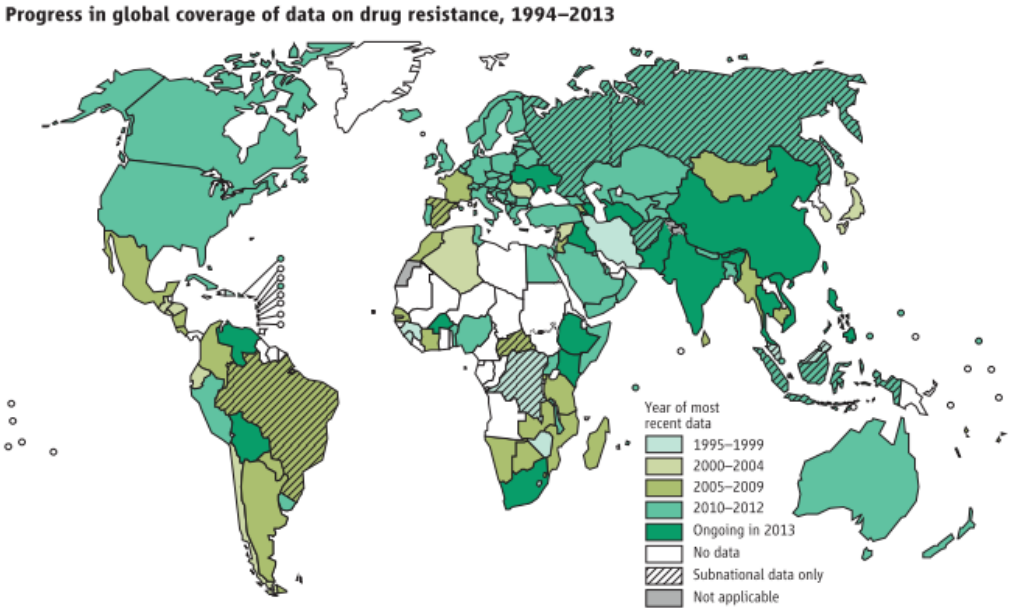

Figure 1-3: Progress in global coverage of data on drug resistance from 1994 – 2013. The map shows the coverage of drug resistance reports and the year of their most recent representative data. Data was collected and illustrated in 2013 by the WHO in their annual multidrug and extensively drug-resistant TB report (WHO 2013).

1.1.2 Pathogenesis and disease

Human infections with tuberculosis are caused by *M. tuberculosis*. It is an endemic disease, with about two billion people infected, and besides HIV/AIDS it is the most common cause of adult death resulting from an infectious agent. (Dye *et al.* 1999; Fox and Menzies 2013; WHO 2013). Two strains infect humans, *M. tuberculosis hominis* and *M. tuberculosis bovis.* Tuberculosis is more frequent in the young and the very old and there are definite racial and ethnic differences in incidence. The disease also flourishes in socially deprived areas and poverty and malnutrition seem to be important predisposing factors. There is a higher risk for men and for people suffering from alcoholism, chronic lung disease and conditions causing immune-suppression, e.g. cancer and AIDS (Fox and Menzies 2013; WHO 2013).

Infection of tuberculosis occurs by inhaling airborne *M. tuberculosis.* In theory, one single bacterium is able to cause an infection. Residual macrophages in the airways engulf the pathogen, which then move into the epithelial layer and trigger a local inflammatory response. Subsequently, mononuclear cells are recruited from

surrounding blood vessels and facilitate bacterial replication by provision of new host cells. This accumulation of infected cells constitutes the origin granuloma, the formation of which is essential for the containment of the bacteria. The infected cells are surrounded by lymphocytes and a fibrous cuff and the containment of the bacteria is assured as long as the host immune response is intact. Changes in the host's immunity lead to caseation of the granuloma centre and subsequent release of the contained bacteria into the airway, which can then be transmitted to other hosts by coughing or spread within the original host (Fig. 1-4) (Russell *et al.* 2010a; Russell 2011).

Based on this, *M. tuberculosis* infection can result in the following outcomes: (1) the minority of exposed people will eradicate the pathogen or develop an active disease, called primary tuberculosis, within two years after exposure. The chances for this outcome are around 5 – 10%, depending on the individual circumstances. (2) The majority of exposed people will develop a so-called latent infection, in which the infected person shows no clinical signs of the disease but develops an effective immune response against the pathogen to control its spread internally. The bacteria are contained within granulomas but are not eradicated. This condition allows the third possible outcome of a tuberculosis infection in which the controlled but not eradicated bacteria can lead to the reactivation of the disease, the so-called post-primary or secondary tuberculosis (Young *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011; Fox and Menzies 2013).

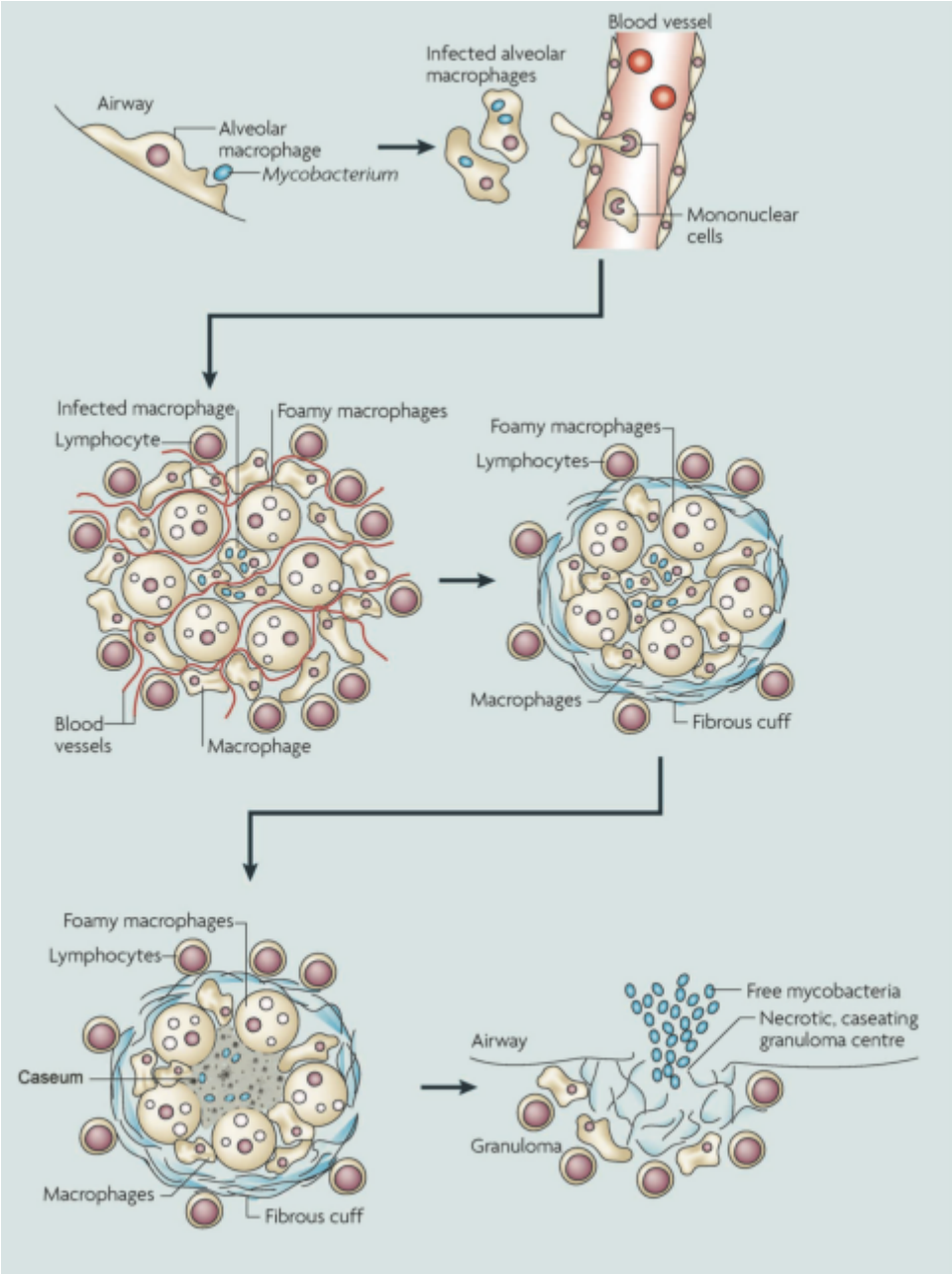

Figure 1-4: The life cycle of *M. tuberculosis* as taken from Russell *et al.* (2010a)

1.1.3 Granulomas

Basically all pathogenic mycobacteria produce granulomas, the hallmark structures of tuberculosis infection. Similar to macrophages, granulomas may play a double role in the infection, both containing it and creating an environment where the bacteria can persist (Ramakrishnan 2004; Barry *et al.* 2009; Young *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011). Granulomas normally begin with an aggregation of mononuclear phagocytes which is surrounded by individual infected macrophages**.**

These macrophages then become activated, during which they are seen to increase in their size and number of subcellular organelles, develop ruffled cell membranes, and have improved phagocytic and microbiocidal efficiencies (Adams 1976; Davis *et al.* 2002; Ramakrishnan 2004; Barry *et al.* 2009; Young *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011; Ramakrishnan 2013)**.** Additional differentiation of the macrophages into epithelioid cells is a common feature of all *Mycobacterium* granulomas. These epithelioid cells have tightly interdigitated cell membranes in zipper-like arrays linking adjacent cells (Adams 1976; Ramakrishnan 2004; Russell *et al.* 2010a; Ramakrishnan 2013). Furthermore, several macrophages can fuse to form giant cells. The pathogenic definition of a granuloma is an organized accumulation of differentiated macrophages with a typical morphology (Adams 1976; Barry *et al.* 2009; Young *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011).

Tuberculosis granulomas can be differentiated into three major groups (Fig. 1-5): (1) The classical granuloma, which can be found in active and latent tuberculosis, (2) non-necrotizing granuloma, which are most frequent in active disease and (3) fibrotic lesion, which are usually common in latent infection (Barry *et al.* 2009).

Human and other mammalian tuberculous granulomas contain a combination of lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components (Flynn and Chan 2001a; Barry *et al.* 2009; Young *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011; Ramakrishnan 2013). The roles of all the

accessory cells in the granuloma have yet to be elucidated, but certain T-lymphocyte (T cell) subsets are critical for maintaining granuloma integrity and restricting bacterial numbers (Flynn and Chan 2001a).

An outstanding characteristic of certain tuberculous granulomas is the presence of areas of caseous necrosis, regions of acellular debris that have a distinct histology (Adams 1976; Ramakrishnan 2004; Barry *et al.* 2009; Ramakrishnan 2013). A perfect 'textbook picture' of a human granuloma would be a central core of necrosis surrounded by epithelioid cells, which are surrounded by a cuff of $CD4^+$ and $CD8^+$ T cells lymphocytes. A circle of peripheral fibroblasts can sometimes be found as a final border between the granuloma and healthy tissue (Ramakrishnan 2004; Barry *et al.* 2009; Young *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011).

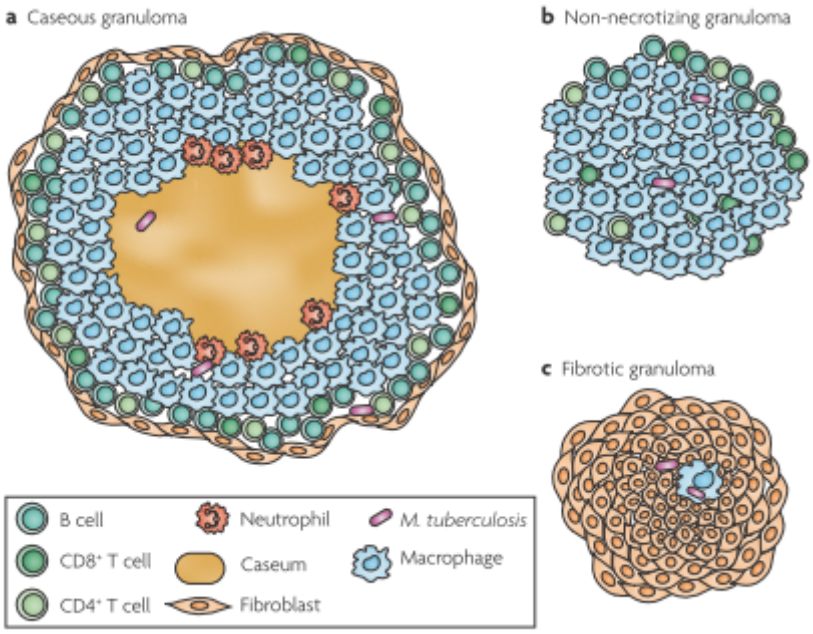

Figure 1-5: Tuberculous granuloma. Overview of the three granuloma types found in mammals: classical tuberculosis granuloma (a), non-necrotizing granuloma (b) and fibrotic granuloma (c) and their cellular constituents taken from Barry *et al.* (2009).

1.1.4 Latency and reactivation

One of the most extraordinary features of *M. tuberculosis* (Mtb) is its ability to create a latent infection. It is estimated that around one-third of the world's population is infected with latent tuberculosis infection (LTBI) (Cardona and Ruiz-Manzano 2004; Barry *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011; Patel *et al.* 2011; Fox and Menzies 2013; WHO 2013). The textbook term latent tuberculosis infection is solely defined on the positive outcome in either a tubercle skin test (TST) or an *in vitro* interferon gamma release assay (IGRA) without the presence of clinical symptoms or isolation of mycobacteria from the host (Barry *et al.* 2009; Mack *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011). To date, only these two methods, TST and IGRAs, are available for testing latent tuberculosis, and neither of them is currently sufficient to be validated as a reliable biomarker to support and enhance contemporary medicine and research. The TST is highly cross-reactive with the BCG vaccine, even years after sensitisation. The IGRAs work independently from the vaccine because the reaction is based on a Mtb specific genomic region. Despite this advancement neither of the tests can distinguish between active and latent disease (Barry *et al.* 2009; Young *et al.* 2009; Lin and Flynn 2010; Pai *et al.* 2010; Cattamanchi *et al.* 2011; Diel *et al.* 2011; Gideon and Flynn 2011; Walzl *et al.* 2011; Rangaka *et al.* 2012).

Latent tuberculosis is the product of a complex set of interactions between the bacterium and host immune response (Chan and Flynn 2004; Lin and Flynn 2010; Gideon and Flynn 2011). The host immune response isolates the bacteria in the granuloma and therefore prevents an outbreak of the disease, whereas the bacteria might change into a dormant life cycle stage which protects them from detection and elimination, a so-called stand-off situation (Flynn and Chan 2001b; Lin and Flynn 2010; Gideon and Flynn 2011).

For a long time, it was accepted that the bacteria which are responsible for reactivation of tuberculosis hide within the granuloma, the hallmark of the disease. Recent findings, however, suggest that tubercle bacteria other than the ones in the granuloma can also be responsible for reactivation. Mycobacterial DNA was detected outside cells and granulomas in latent infected tissue samples (Neyrolles *et al.* 2006; Young *et al.* 2009), but the direct habitation of the bacteria within the host and its physiological state is still unknown, which complicates the development for diagnostic methods and vaccines (Lin and Flynn 2010; Gideon and Flynn 2011; Patel *et al.* 2011).

The conventional model of tuberculosis is simply the division of the active or the latent disease without the consideration of any overlap (Barry *et al.* 2009; Young *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011). In recent years, more studies support the idea that latent tuberculosis is a dynamic process and the individual state of the host can differ between a complete clearance of the bacteria to inhabiting active replicating bacteria in the absence of clinical signs (Barry *et al.* 2009; Young *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011).

Reactivation of latent infection can happen anytime during the lifecycle and supports the assumption that latency is a dynamic process. There are a variety of factors which can initiate reactivation and lead to the establishment of an active disease. Currently, two mechanisms are known leading to reactivation: (1) defects in $CD4^+$ T cell production, which has been discovered in connection with infection of HIV, but other factors can also lead to reactivation (Barry *et al.* 2009; Ahmad 2010; Lin and Flynn 2010; Russell *et al.* 2010a; Gideon and Flynn 2011; Ernst 2012) and (2) neutralisation of TNF (Tumor Necrosis Factor) as found in therapeutic TNF neutralizing treatments (Barry *et al.* 2009; Lin and Flynn 2010; Russell *et al.* 2010a; Gideon and Flynn 2011; Ernst 2012). Moreover other medical conditions like diabetes, T cell exhaustion, altered antigen expression or cell trafficking have been associated with reactivation, but the underlying mechanisms have not been fully eliminated (Ernst 2012).

The major common ground in all reactivation cases is the suppression or weakening of the immune system. Conditions which cause the suppression of the immune system are multifaceted and can vary from something as simple as aging or chronic stress, over malnutrition, and medical conditions that compromise the immune system such as poorly controlled diabetes mellitus to renal failure and therapy with immunosuppressive drugs (Lin and Flynn 2010; Gideon and Flynn 2011; Fox and Menzies 2013).

1.1.5 Current model systems

Difficulties in studying *M. tuberculosis* include the slow growth rate, *M. tuberculosis* has a generation time of about 24 hours (h), the necessity of working in high containment biosafety level-3 (BLS-3) facilities, and expensive and non-ideal mammal hosts (Broussard and Ennis 2007; Tobin and Ramakrishnan 2008).

Scientists have sought substitute mycobacterial models for *M. tuberculosis*. The ideal surrogate model hosts for human tuberculosis have to be susceptible for to infections of *M. tuberculosis* or a closely related bacterium, which produces a comparable chronic disease, and have to be genetically tractable as well (Broussard and Ennis 2007). An overview of existing model organisms and which state of tuberculosis they can mimic is given in Fig. 1-6.

Traditionally, mammalian models (e.g. mouse) and cell cultures have been used to investigate the *Mycobacterium* – host interactions and can be used in a variety of ways to understand tuberculosis. The most commonly used experimental model for human tuberculosis is the mouse model. Mice are routinely used to work on current drugs and vaccines. They have also been used to understand key aspects in the pathology, immunology and genetics of the disease. The variety of available mutants and a diverse genetic background have helped to understand the influence and importance of certain parts of the immune system by removing them. The big disadvantage of this model is that the mouse is not a natural host for mycobacteria and

therefore the outcome of the disease and the granuloma formation differs in this model compared to humans (Cosma *et al.* 2003; Pozos and Ramakrishnan 2004; Gupta and Katoch 2005; 2009; Young 2009; Patel *et al.* 2011).

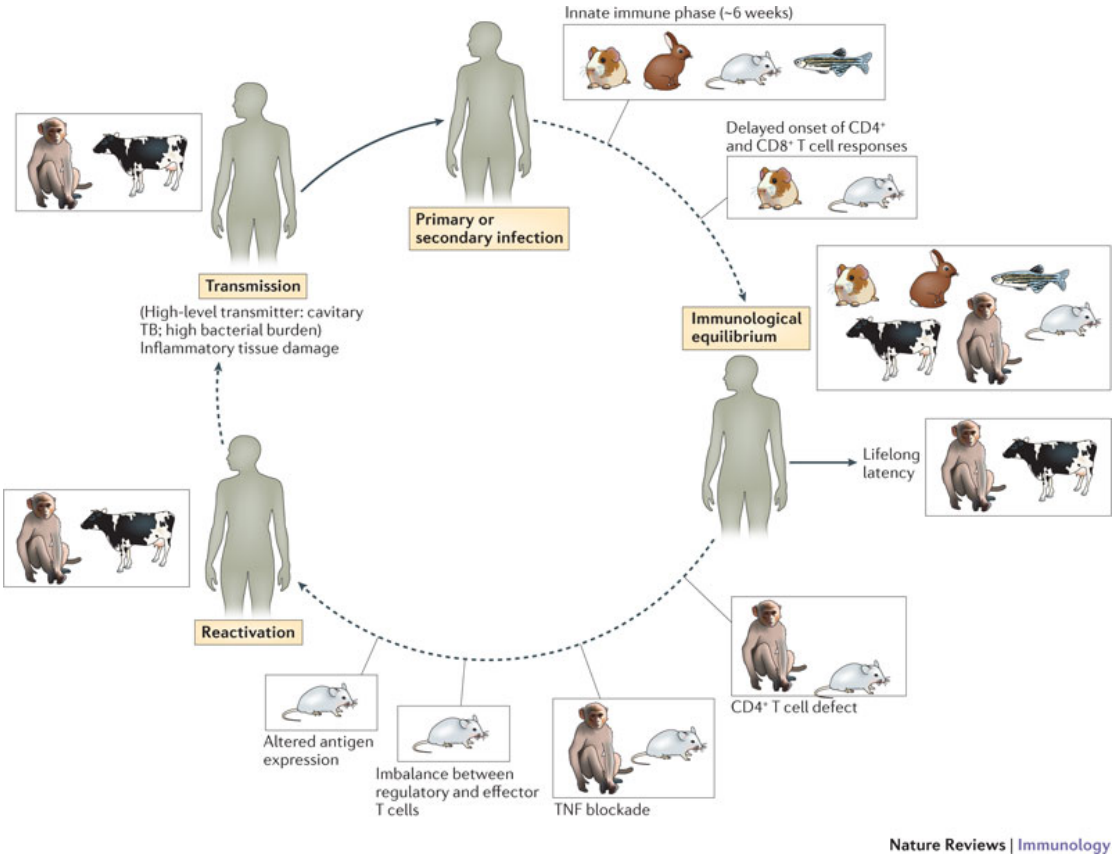

Figure 1-6: Stages of the immunological life cycle of human tuberculosis that can currently be modelled in experimental animals as shown in Ernst, 2012

Guinea pigs and rabbits were also used as host organisms, but the higher cost in comparison to mice and the lack of immunological reagents limits their use as model animals. Furthermore, guinea pigs are exquisitely susceptible to progressive pulmonary infections, while most rabbits are resistant to *M. tuberculosis* infections and can be used to differentiate between *M. tuberculosis* and *M. bovis* infections (Cosma *et al.* 2003; Gupta and Katoch 2005; Padilla-Carlin *et al.* 2008; Gupta and Katoch 2009; Young 2009). Recent studies disagree and suggest that guinea pigs and rabbits might be useful for modelling latent tuberculosis (Lenaerts *et al.* 2007; Manabe *et al.* 2008; Patel *et al.* 2011).

Non-human-primates, like macaques, are currently the only model for natural transmission, especially since macaques infected with *M. tuberculosis* show similar lesions to those in humans and most human immunology reagents can be used on them as well. Furthermore, it is the only model that seems to produces latent infections similar to those in humans. Besides all the advantages, the non-human-primate model is expensive and highly criticised by the majority of people and therefore used as proof-of-concept in drug and vaccine trials (Gupta and Katoch 2005; 2009; Young 2009; Mattila *et al.* 2011; Patel *et al.* 2011).

In recent years, new model hosts for tuberculosis have been drawn in comparison, e.g. amoeba (*Dictyostelium discoideum*), fruit flies (*Drosophila* spp.), leopard frog (*Rana pipiens*), goldfish (*Carassius auratus),* medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). Since the discovery of the close relationship of *M. tuberculosis* with *M. marinum* a wide range of new modelling possibilities have become available due to the fact that *M. marinum* is a natural pathogen of a variety of non-mammalian hosts (Parisot 1958; Ramakrishnan *et al.* 1997; Haegele *et al.* 2000; Cosma *et al.* 2003; Dionne *et al.* 2003; Swaim *et al.* 2006; Hagedorn *et al.* 2009; Clarke 2010; O'Callaghan and Vergunst 2010).

The *Dictyostelium* model allows the genetic manipulation of the host to identify host determinants of *Mycobacterium* – macrophage interaction (Haegele *et al.* 2000; Cosma *et al.* 2003; Hagedorn *et al.* 2009; Clarke 2010) and the *Drosophila* model offers the potential to employ genetics to study the contribution of innate immunity (Cosma *et al.* 2003; Dionne *et al.* 2003).

Goldfish, zebrafish and medaka turned out to be good models for studying human tuberculosis. They are genetically tractable, having an innate and adaptive immunity, and embryos and some mutants are transparent (Talaat *et al.* 1998; Cosma *et al.* 2003; Pozos and Ramakrishnan 2004; Broussard and Ennis 2007). The zebrafish in particular seems to be a new star on the model horizon.

1.2 *Mycobacteriosis*

1.2.1 Genus Mycobacterium

Mycobacteria belong to the genus *Mycobacterium* (Family *Mycobacteriaceae*), which consists of 54 species in total (Eisenstadt and Hall 1995). Mycobacteria differ greatly in their ecology, from the obligate pathogen *M. tuberculosis*, a leading cause of human mortality worldwide, to saprophytic soil residents such as *Mycobacterium terrae* (Gauthier and Rhodes 2009; Hagedorn and Soldati 2009).

Classification of species is complex and mycobacteria can be grouped according to a number of different factors, e.g. growth rate, pigmentation, fatty acid content and pathogenicity (Belas *et al.* 1995).

The first way to classify mycobacteria is based on their pathogenicity, and this differentiates them into two groups: tuberculosis and non-tuberculosis mycobacteria (Eisenstadt and Hall 1995). The *M. tuberculosis* complex, *M. leprae* and some atypical mycobacterial species have been reported to cause mycobacterial disease in man, e.g. tuberculosis or leprosies (Wayne and Sramek 1992).

The non-tuberculosis mycobacteria can be further grouped into slow and rapidly growing mycobacteria. Fast growers like *M. fortuitum* require less than 7 days to produce colonies on solid agar, while slow-growing species, e.g. *M. marinum* may require weeks to month to achieve comparable growth (Lévy-Frébault and Portaels 1992). The first classification of mycobacteria was sub-divided into 4 groups (three slow-growing and one rapidly growing) (Runyon 1959): (1) photochromogenic (from pigment in response to light), (2) scotochromogenes (from pigment in the absence of light), (3) non-chromogenic (do not produce pigments) and (4) rapidly growing mycobacteria. Beyond these distinctions, the nomenclature of *Mycobacterium* spp. is still in a state of flux.

Mycobacteria are Gram-positive, aerobic, acid-fast, non-motile bacteria and have pleomorphic rods. They are approximately 0.3 - 0.7 µm wide by 14 µm long

(Belas *et al.* 1995) and possess a unique and therefore characteristic cell wall. The main composition consists of arabinogalactan, a polysaccharide comprising of arabinose and galactose, which is connected on one site to peptidoglycan and to fatty acids and the mycolic acids on the other side. Mycolic acids are dendric 3-hydroxyacids, which are substituted by aliphatic chains in the 2- and 3-position. These chains are 60 – 90 carbon-atoms long and are responsible for mycobacteria's acid-fastness (Lederer 1971; Ducati *et al.* 2006; Welin 2011). This structure is the basic skeleton of the mycobacterial cell wall and is referred to as the mycolylarabinoga-lactan-peptidoglycan complex (Ducati *et al.* 2006).

Additionally, several lipoglycans are located in the cell wall, anchored in the plasma membrane and outreach till outside of the exterior of the cell wall. These lipoglycans include lipoarabinomannan (LAM) and its pre-stages lipomannan (LM), and phosphatidyl-myo-inositol mannosides (PIM) (Ducati *et al.* 2006; Kleinnijenhuis *et al.* 2011; Rajni and Meena 2011; Saiga *et al.* 2011; Welin 2011). LAM or to be more precise the capping of LAM appears to have a significant influence on the virulence of a mycobacterial strain. Depending on the *Mycobacterium* species, the LAM is capped with either mannose (ManLAM), phospho-myo-inositol (PILAM) or is uncapped (AraLAM) (Dao *et al.* 2004; Rajni and Meena 2011; Welin 2011). Slow-growing and virulent strains like *M. tuberculosis, M. bovis* and *M. marinum* possess ManLAM while fast-growing and non-virulent strains like *M. smegmatis* or *M. chelonae* exhibit PILAM or AraLAM. Furthermore some species like *M. tuberculosis* and *M. marinum* exhibit a 19 kilo Dalton (kDa) lipoprotein whose function is still not quite clear (Ciaramella *et al.* 2004; Paddock *et al.* 2006; Welin 2011). The type of capping has an influence on the immune response. While ManLAM appears to induce an anti-inflammatory response and inhibits the production of TNFα and IL- 12, PILAM and AraLAM trigger the production of other cytokines like TNFα, IL 1α, IL-1β, IL-6, IL-8, and IL-10. In particular, the inhibitory function of ManLAM appears to facilitate a prolonged survival of bacteria in the host cell (Nigou *et al.* 2002; Rajni and Meena 2011).

1.2.2 Mycobacteria from fish

Mycobacteria are responsible for a number of diseases in a wide range of hosts, including mammals and aquatic animals. The first report of mycobacteriosis in cold–blooded animals was by Bataillion *et al*. in 1897 in carp (Gauthier and Rhodes 2009). Since its initial discovery, Nigrelli and Vogel (1963) reported that Mycobacteriosis occurs worldwide in more than 40 families and 151 species of marine and freshwater fish (Nigrelli and Vogel 1963; Gauthier and Rhodes 2009; Lewis and Chinabut 2011). Other susceptible animals include oysters, shrimps, crayfish, frogs, lizards, turtles, as well as mammals, including man. To date, there are 54 official mycobacterial species and some still remain to be identified (Collins *et al.* 1984; Lewis and Chinabut 2011).

Mycobacteria are widespread in the environment, particularly in aquatic reservoirs. Many bacteria of the genus *Mycobacterium* have been reported to cause mycobacteriosis in fish. In 1979, Dunlin reported numerous mycobacteria that were pathogenic to fish species including different species which nowadays have been reclassified to *M. marinum* and *M. fortuitum*, which are the two most important species causing mycobacteriosis in fish and humans. Other species associated with the disease have also been reported, e.g. *M. chelonae, M. neoaurum*, *M. salmoniphilium, M. simiae* and *M. scrofulaceum* (Gauthier and Rhodes 2009)..

M. marinum was first recognized 1926 by Aronson and later identified by Linell and Norden (1954) as a human pathogen (Linell and Norden 1954; Collins *et al.* 1984; Decostere *et al.* 2004). It causes systemic tuberculosis in fish, reptiles and amphibians (Post 1987; Greer *et al.* 2003; Ramakrishnan 2013) and forms the largest proportion of all mycobacteria in fish (Decostere *et al.* 2004). *M. marinum* is ubiquitous and is found worldwide in bodies of fresh water, brackish water and salt water. Tropical freshwater and marine fish are susceptible to *M. marinum*. Despite the fact that infections in temperate and cold water have been described, *M. marinum* has traditionally been

regarded as a warm-water pathogen (Gauthier *et al.* 2008). *M. marinum* are described as moderate to long rods, with frequent cross-barring. The colonies are smooth to rough and non-pigmented when grown in the dark. When they are grown in the light or exposed to light when they are young, they become brilliant yellow (photochromogenic). *M. marinum* is slow growing, taking 7 days or more to grow at 30°C. Optimum growth temperature ranges from 25°C to 35°C, but *M. marinum* can adapt to growth at 37°C (Belas *et al.* 1995).

M. fortuitum, isolated from fish, is less frequently documented than *M. marinum* (Decostere *et al.* 2004). It was first isolated in 1953 and is not highly host-specific with regards to temperature or salinity (Ross and Brancato 1959). Gordon and Mihm reported that *M. fortuitum* grows on MacConkey agar and changes the indicator colour, but does not utilise oxalate or mucate (Gordon and Mihm 1959). Thus, *M. fortuitum* differs from the other mycobacteria. The bacterium can either be coccoid, or short to long rods. The colonies are usually off-white or cream, or when they are grown in medium containing malachite green they may absorb the green dye. Their optimum growth temperature ranges from 30°C to 37°C (Belas *et al.* 1995).

M. chelonae does not cause infections in humans, but it represents the third species of mycobacteria alongside *M. marinum* and *M. fortuitum*, which dominate the literature of fish diseases (Gauthier *et al.* 2008). *M. chelonae* was originally isolated from a turtle in 1903 by Friedman and was later only found in cold water salmonid species (Belas *et al.* 1995; Bruno *et al.* 1998; Greer *et al.* 2003; Decostere *et al.* 2004). Infections of *M. chelonae* can further persist through fresh and salt water phases in the fish life cycle (Frerichs 1993). *M. chelonae* is a pleomorphic organism ranging from long narrow to short thick rods, but coccoid forms are also reported. The colonies may be smooth, moist and shiny or rough, and they are usually nonchromogenic to creamy buff colour. Their optimum growth temperature ranges from 22°C to 40°C (Falkinham 1996).

In recent years another *Mycobacterium* strain, *M. salmoniphilum*, a close relative to *M. chelonae*, is coming to the centre of attention of aquaculture research (Whipps *et al.* 2007a). This specific strain is causing mycobacteriosis in Atlantic salmon (*Salmo salar*) (Zerihun *et al.* 2011; Aro *et al.* 2013; Pourahmad and Nemati 2013), rainbow trout (*Oncorhynchus mykiss*) (Pourahmad and Nemati 2013) and Atlantic cod (*Gadus morhua*) (Zerihun *et al.* 2012), which are fish species of high commercial importance and diseases affecting these stocks are therefore important for aquaculture research.

Detection of mycobacteriosis in fish normally follows after disease outbreaks have been reported and comprises histological and quantitative bacteriology. Further, a variety of molecular tests are commercially available such as GenType *Mycobacterium* assay, INNO-LiPA *Mycobacterium* assay and AccuProbe *Mycobacterium* assay to characterise the pathogen (Pourahmad *et al.* 2009). The assays are based on the detection of mycobacteria from bacterial cultures grown from infected fish or other host organisms, which depending on the mycobacteria species, can take weeks to grow. Based on this, this can be a very time consuming detection method (Ruiz *et al.* 2002; Lebrun *et al.* 2003; 2005). More recently, specific PCR primers and techniques have been developed to detect and characterise mycobacteria in fish on a more specific and sensitive level (Pourahmad and Richards 2013; Pourahmad *et al.* 2014). However, these techniques still require samples taken from dead or sacrificed fish. New detection methods are needed to determine mycobacterial infections in fish by non-lethal methods, e.g. in blood samples.

1.2.3 Epidemiology

Mycobacteriosis is one of the most common diseases affecting cultured and wild fish worldwide (Frerichs 1993; Belas *et al.* 1995; Hagedorn and Soldati 2009). Around 151 fish species are affected by mycobacteriosis, caused by 40 representative families (Nigrelli and Vogel 1963; Gauthier and Rhodes 2009).

Since 1963 the number of affected fish and pathogenic mycobacteria reported has expanded considerably, and infections caused by mycobacteria have been described in freshwater and marine fishes from tropical to subarctic latitudes (Arakawa and Fryer 1984; Rhodes *et al.* 2004).

There have been very few reports on the occurrence of mycobacteriosis in the wild and very little is known about its prevalence and impact on wild fish (Heckert *et al.* 2001).

Transmission of mycobacteria is barely understood. Water and biofilms are natural habitats for mycobacteria (Pedley and Bartram 2004), so it seems like the transmission is water-based.

Fish can be affected by the intake of contaminated food or water, cannibalism of infected fish, contaminated aquatic detritus or entry via injuries, skin abrasions or external parasites (Post 1987; Frerichs 1993; Belas *et al.* 1995; Decostere *et al.* 2004; Hagedorn and Soldati 2009; Noga 2010). Mycobacteriosis lesions occurring along the gut or in the skin and gills may be responsible for the release of pathogens into the water. Release of infective bacteria from other organs is ineffectual until the fish dies and disintegrates (Decostere *et al.* 2004; Gauthier and Rhodes 2009).

Vertical transmission of mycobacteria has been suggested (Gauthier and Rhodes 2009), but not yet been documented (Decostere *et al.* 2004; Gauthier and Rhodes 2009) and trans-ovarian transmission in viviparous fish has also been reported (Frerichs 1993; Gauthier and Rhodes 2009).

Although there is no certain evidence to confirm that environmental stress can cause mycobacteriosis in fish, some culturing circumstances may promote the disease (Giavenni *et al.* 1980; Daoust *et al.* 1989). Daoust *et al*. (1989) suggest that mycobacteriosis in yellow perch (*Perca flavesceus*) held in an aquarium, occurred due to a depressed immune response in these fish.

Temperature requirements of the host organism are also important in the spread of mycobacteriosis (Ramakrishnan and Falkow 1994). For example, fish

affected with *M. chelonae* have a higher mortality rate at 18°C compared with 12°C (Arakawa and Fryer 1984).

Other aquatic vertebrates may transmit mycobacteriosis to fish. Frogs, snakes and turtles could be part of the transmission cycle. Other invertebrates, e.g. water fleas, have been shown to play a role in the transmission of mycobacteria (Decostere *et al.* 2004).

1.2.4 Pathogenesis and disease

Mycobacteria are omnipresent in the environment, and both humans and fish are susceptible to these pathogens. Despite this, the pathogenesis of non-tuberculosis mycobacterial infections has been poorly characterized (Talaat *et al.* 1999). Mycobacteria, whether infectious to fish or mammals, are facultative intracellular bacterial pathogens (Pasnik *et al.* 2003). They are able to obtain nutrients and escape the immune system by growing within host cells (see Section 1.3.) (Amer and Swanson 2002).

M. marinum, for example, causes systemic infections, similar to human tuberculosis, in fish, amphibians, and other ectotherms, and survives and replicates in host macrophages, where it prevents phagosome maturation (Rybniker *et al.* 2003; Solomon *et al.* 2003; Hagedorn and Soldati 2009). It has been shown that phagosomes containing *M. marinum* bacterial cells do not fuse with lysosomes (Barker *et al.* 1998; Decostere *et al.* 2004).

Piscine mycobacteriosis is, in most cases, a chronic progressive disease (Hedrick *et al.* 1987) that does not show any clinical signs or may take years to develop into a clinically noticeable illness (Austin and Austin 2007). All kinds of tissues can be involved, from eyes to musculature and fins. Acute disease is associated with high bacterial loads and is observed occasionally (Whipps *et al.* 2008).

External clinical signs of chronic mycobacteriosis include scale loss, dermal ulceration, pigmentary changes, abnormal behaviour, spinal defects, emaciations and

ascites, but these are non-specific (Gauthier and Rhodes 2009). Gross internal signs of this infection include enlargement of the spleen, kidney and liver. Additionally, grey or white nodules can appear in internal organs, which are characteristic for mycobacteriosis (Chinabut 1999).

The classical histopathological symptom of mycobacteriosis is granulomatous inflammation. Generally, fish mycobacteriosis is a systemic disease with granulomas produced in multiple organs or tissues as in human tuberculosis. Significant variations in the size and structural organization of granulomas are seen, from highly organized lesions with thick epithelioid layers to poorly organized inflammation with minimal epithelioid cell formation. Caseous necrosis and/ or calcification of the core region may be observed in a manner similar to the human tuberculosis.

In summary, *M. marinum* infections of frogs and fish bear some similarities to human *M. tuberculosis* infection. Fish granulomas caseate and give the opportunity to study this process, whereas frog granulomas maintain a chronic infection with a stable number of organisms in a manner similar to some humans and may be useful for the examination of the bacteria–host interface during a long-term smouldering infection (Ramakrishnan 2004).

1.2.5 Human infection

Mycobacteriosis in humans leads back to exposure of persons to mycobacterial contaminated water. Breaks in the skin serve as an entry point for the organisms. Large outbreaks have generally been connected with an outbreak in a swimming pool (Falkinham 1996). Tuberculosis infections in people who use public swimming pools appeared in 1939 in Sweden and in the early 1950s in the United States, but no causative agent could be identified until 1954 (Collins *et al.* 1984; Gluckman 1995). Linell and Norden identified an acid-fast bacterium as the infection source in 1954 after 80 people who had used the same public swimming pool were diagnosed with cutaneous granulomatous lesions (Linell and Norden 1954; Collins *et al.* 1984;

Gluckman 1995). The identified organism was named *Mycobacterium balnei*, after the Latin word for bath, which appeared to be synonymous to *M. marinum.* Because of these early reports of human disease which are associated with swimming pools, the disease has been called “swimming pool granuloma” (Belas *et al.* 1995; Gluckman 1995). Swimming pool granuloma has essentially disappeared because of the proper chlorination of public swimming pools (Gluckman 1995), although other aquatic environments, including fresh, salt and brackish water, have been sources of human mycobacteriosis (Belas *et al.* 1995; Gluckman 1995).

Nowadays, most infections occur in people who keep an aquarium at home, but infections may also be an occupational risk for certain professionals, such as aquaculturists, fish processors, or pet shop workers (Belas *et al.* 1995; Gluckman 1995; Falkinham 1996). Basically, any water related activity is a potential risk (Belas *et al.* 1995; Gluckman 1995; Falkinham 1996; Hagedorn and Soldati 2009; Ramakrishnan 2013).

Due to the optimal growth temperature of 30°C – 32°C, infections of mycobacteriosis are localized primarily in the skin and are less commonly in deeper tissues. The infection can have several different clinical presentations but the most common is a solitary papulonodular lesion on an extremity. In particular, these lesions tend to arise over a prominence that has a tendency to be abraded, such as a finger, hand or knee (Gluckman 1995). The incubation period is relatively long, ranging from 2 – 6 weeks. The visual infection begins with a little red papule that slowly enlarges into violaceous nodular plaques. Sometimes these lesions can be more pustular or even ulcerate, but they are painless. There are no associated systemic symptoms and restricted adenopathy is uncommon. Thus, there is often a long time between onset of the lesion and recognition by the host and treatment. Deep infections can lead to a variety of diseases, like arthritis or carpal tunnel syndrome. These are the result of extensive cutaneous infection or direct inoculation of the bacterium (Gluckman 1995).

In some cases, treatment includes surgery and in the worst case amputation. Because of that, piscine mycobacteria constitutes a double threat to the management of fish in aquaculture: first by causing morbidity and mortality in fish and second by endangering the people who work with the infected animals (Belas *et al.* 1995).

1.3 *Mycobacteria – Host – Interactions*

1.3.1 Recognition by the host

Several receptors on the surface of macrophages recognize pathogenic bacteria. The receptors can be divided into the recognition of opsonized and non-opsonized bacteria. The complement system is the major humoral non-specific defence mechanism that consists of multiple proteins and is responsible for opsonisation (El-Etr and Cirillo 2001; Mason and Ali 2004; Hossain and Norazmi 2013). In addition, an activated complement system can lead to increased vascular permeability, recruitment of phagocytic cells and lysis of bacteria (Mason and Ali 2004). Non-opsonized mycobacteria can bind directly to complement receptor 3 (CR3) and CR4 (Andersen 1997; El-Etr and Cirillo 2001; Pieters 2001; van Crevel *et al.* 2002; van de Vosse *et al.* 2004; Vergne *et al.* 2004). The best described receptor for recognition of non-opsonized mycobacteria is the macrophage mannose receptor (MR), which recognizes terminal mannose residues on mycobacteria (Andersen 1997; El-Etr and Cirillo 2001; Pieters 2001; van Crevel *et al.* 2002; van de Vosse *et al.* 2004; Vergne *et al.* 2004; Ottenhoff *et al.* 2005; Kleinnijenhuis *et al.* 2011; Hossain and Norazmi 2013).

Besides the complement and mannose receptors, macrophages have also Fc receptors, scavenger receptors and Toll-like receptors (TLRs) (Cooper *et al.* 2011; Kleinnijenhuis *et al.* 2011; Hossain and Norazmi 2013). The TLRs play a central role in immune recognition in the context of the cluster of differentiation 14 (CD 14) and recognize broad molecule patterns called pathogen associated molecular pattern

(PAMPs) on infectious agents. In the case of mycobacteria, TLRs recognize the major mycobacterial cell component LAM which appears to resemble that of gram-negative bacterial lipopolysaccharide (LPS) (van Crevel *et al.* 2002; Mason and Ali 2004; Orme 2004; van de Vosse *et al.* 2004; Vergne *et al.* 2004; Ottenhoff *et al.* 2005; Cooper *et al.* 2011; Kleinnijenhuis *et al.* 2011; Hossain and Norazmi 2013; Torrado and Cooper 2013). Further collectins, surfactant protein A (Sp-A) and mannose-binding lectins (MBLs) are involved in the uptake of mycobacteria in macrophages (van Crevel *et al.* 2002; Vergne *et al.* 2004).

1.3.2 Host defence mechanism against mycobacteria

Binding to one of the macrophage receptors results in phagocytosis and the release of inflammatory cytokines by the phagocyte (van Crevel *et al.* 2002; Mason and Ali 2004). Macrophages exhibit various degrees of anti-mycobacterial activity, like the reactive oxygen intermediates (ROI) that are generated by macrophages via oxidative burst or the production of reactive nitrogen intermediates (RNI). The RNI production in macrophages is induced by interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) via the action of the inducible form of nitric oxide synthase (NOS2). The proposed effect of all bactericidal activity mechanisms is the eradication of the bacteria (Andersen 1997; Bogdan *et al.* 2000; Flynn and Chan 2001a; Chakravortty and Hensel 2003; Co *et al.* 2004; Mason and Ali 2004; Welin 2011; Parikka *et al.* 2012; Torrado and Cooper 2013).

Macrophages play a key role in both the establishment of immunity to mycobacteria as well as the maintenance of the immune reaction. They are the first responder to encountered mycobacteria and are capable of initiating an early immune response (Mason and Ali 2004; van de Vosse *et al.* 2004; Young *et al.* 2008). Dendritic-like cells also play an important role in mammals because they are the classic antigen-presenting cells (APC) in infection with mycobacteria (Mason and Ali

2004; Orme 2004; van de Vosse *et al.* 2004; Ottenhoff *et al.* 2005; Young *et al.* 2008; Cooper *et al.* 2011; Torrado and Cooper 2013).

Once APCs have processed the ingested mycobacteria or mycobacterial protein, they present the antigen in the context of the major histocompatibility complex (MHC) class II surface molecules to naive $CD4^+$ T-lymphocytes. This is one of the initial steps in the development of acquired specific immunity to the mycobacteria. The APCs produce Interleukin-12 (IL-12), a crucial cytokine to control mycobacteria, to shift the immune reaction to T helper lymphocytes 1 (Th1) and IL-1. This stimulates the $CD4^+$ to produce IL-2 as well as up-regulate lymphocyte IL-2 receptor. The result is a quick clonal expansion of specific $CD4^+$ Th1 lymphocytes or cytotoxic lymphocytes (Tc1 $CD8^+$), which produce the cytokine IFNγ which subsequently actives the macrophage and increases therefore its bactericidal activity. The IFNγ has been shown to be interdependent with other cytokines produced after exposure to mycobacteria, in particular TNFα (Andersen 1997; Flynn and Chan 2001a; van Crevel *et al.* 2002; Co *et al.* 2004; Mason and Ali 2004; van de Vosse *et al.* 2004; Ottenhoff *et al.* 2005; Cooper *et al.* 2011; Green *et al.* 2013; Torrado and Cooper 2013).

The TNFα plays an important role in containing mycobacteria. It serves to acutely enhance the inflammatory response to limit mycobacterial proliferation and maintain the structure and integrity of the granuloma in later phases of infections. (Andersen 1997; Flynn and Chan 2001b; van Crevel *et al.* 2002; Co *et al.* 2004; Mason and Ali 2004; van de Vosse *et al.* 2004; Ottenhoff *et al.* 2005; Torrado and Cooper 2013).

Besides Th1 lymphocytes, there exist also Th2 lymphocytes. The role of these helper T lymphocytes in mycobacterial infections is controversial. They produce Interleukin 10 (IL-10), which is up regulated after mycobacterial infection and decrease the IFNγ production (Flynn and Chan 2001b; van Crevel *et al.* 2002; Mason and Ali 2004). IL-10 and tumor growth factor beta (TGFβ), which is also produced by such T cells, can also inhibit $CD4^+$ proliferation. Production of these cytokines is not limited to

Th2 lymphocytes; all CD4 subsets including macrophages and neutrophils are able to produce them. Release of IL-10 during mycobacterial infection might not be solely a response by the host in order to avoid or limit host-mediated pathology; mycobacteria are able to exploit the host-derived defences to establish a chronic infection (Redford *et al.* 2011; Torrado and Cooper 2013). Interleukin 4 (IL-4) has also been shown to be produced by Th2 lymphocytes. Whether host or mycobacterium, or both, are responsible for the cytokine balance is unclear (Flynn and Chan 2001b; van Crevel *et al.* 2002; Mason and Ali 2004).

Macrophages also produce chemokines during mycobacterial infections, e.g. Interleukin 8 (IL-8), monocyte chemo-attractant protein1 (MCP-1) and RANTES which play roles in inflammatory cell recruitment to infected tissue and in antimycobacterial activity. Numerous chemokines are up-regulated at the site of infection. Inhibition of chemokines could be followed by insufficiency in local tissue response (Andersen 1997; Flynn and Chan 2001b; van Crevel *et al.* 2002; Mason and Ali 2004).

Other subsets of lymphocytes may also play a role in defence against mycobacteria, e.g. γδ T lymphocytes (Cooper and Flynn 1995; Mason and Ali 2004). Comprehensive reviews about the role of cytokines in tuberculosis infections are summarized by Cooper *et al*. (2011) and Torrado and Cooper (2013).

Despite a functional immune response, viable mycobacteria usually persist in the tissue and replicate over the next 2-3 weeks, until specific lymphocytes have been produced by the adaptive immune system (Russell 2011; Ernst 2012; Shaler *et al.* 2012).

1.3.3 Survival of mycobacteria in the host

Despite this organized immune response, some mycobacteria are able to survive in the host. A variety of hypotheses are discussed concerning the survival of mycobacteria in the host, which lead to the latency status.

Avoidance of phagolysosomal fusion is thought to be an important bacterial survival mechanism (Ramakrishnan 2004). Pathogenic mycobacteria are phagocytosed by host macrophages, but the macrophages fail to eradicate the mycobacteria (Flynn and Chan 2001b; Tobin and Ramakrishnan 2008; Russell *et al.* 2010b). Like other pathogenic bacteria, live mycobacteria prevent fusion of the phagosome with the lysosome, and thus survive within the cell (Barker *et al.* 1998; El-Etr and Cirillo 2001; El-Etr *et al.* 2001; Stamm and Brown 2004). In particular, mycobacteria were found to exclude specific lysosomal markers such as cathepsin D and the vaculor-ATPase (Barker *et al.* 1998; Stamm and Brown 2004; Tobin and Ramakrishnan 2008). These mechanisms are suggested to protect pathogenic mycobacteria from phagolysosomal-mediated killing (Tobin and Ramakrishnan 2008).

Another way of avoiding the fusion of the phagolysosome was been identified in a few mycobacteria species which until now has not been detected in other mycobacterial species. *M. marinum* is able to escape from the phagosome into the cytoplasm by initiation of an actin-based motility, which is used during the early stages of infection, to cross epithelial barriers. During granuloma formation this feature is missing (Stamm *et al.* 2003; Stamm and Brown 2004; Tobin and Ramakrishnan 2008). Stamm and Brown (2004) suggested that this actin tail might be an evolutionary adaptation to the aquatic environment, and because of that it is missing in *M. tuberculosis*, *M. ulcerans* or other mycobacterial species.

Another hypothesis suggested that mycobacteria tolerate sub-optimal conditions and survive in acidified compartments in exchange for access to deeper tissues, where they result in granuloma formation (Ramakrishnan 2004; Stamm and Brown 2004; Clay *et al.* 2007; Kanther and Rawls 2010). Consistent with this, there are specific genes in mycobacteria, which are only activated upon aggregation of infected macrophages (gags = granuloma activated genes) (Stamm and Brown 2004). Recent studies have shown that granulomas benefit the infecting bacteria by facilitating their expansion and dissemination. Davis and Ramakrishnan (2009) suggested that the

formation of granulomas act as a tool by the bacteria to expand infection. Mycobacteria-infected macrophages residing in granulomas recruit uninfected cells, which then phagocyte the dying infected cells and subsequently become infected. These newly infected cells can spread and seed new granuloma (Bold and Ernst 2009; Davis and Ramakrishnan 2009; Kanther and Rawls 2010).

Mycobacteria adapt to the stressful conditions which the infected host creates against them. By decreasing their metabolism or switching to a dormant state, the bacilli can tolerate these conditions and appear silent to the immune system (Parrish *et al.* 1998; Cardona and Ruiz-Manzano 2004). Because of this feature, the host immune response is able to control the infection, but fails to kill the pathogen (Chan and Flynn 2004; Co *et al.* 2004).

In addition, it has been proven that mycobacteria can manipulate the host's immune response from the moment of first contact (Shafiani *et al.* 2010; Ernst 2012; Shaler *et al.* 2012; van der Vaart *et al.* 2012).

1.4 *Immune response of fish*

From the physiological point of view, the immune system of fish is quite similar to the mammalian system but there are some differences which have to be considered. The most obvious difference is that fish lack bone marrow and lymph nodes. Instead, lymphoid organs including kidney, thymus and spleen, and mucosa-associated lymphoid tissues take over this important role in fish (Press and Evensen 1999; Tort *et al.* 2003; Alvarez-Pellitero 2008; Meeker and Trede 2008; Uribe *et al.* 2011; Reyes-Cerpa *et al.* 2013).

The kidney is one of the major lymphoid organs and the largest site of haematopoiesis in the teleost fish (Zapata *et al.* 2006; Uribe *et al.* 2011; Reyes-Cerpa *et al.* 2013). The main populations of cells which can be found in the kidney are lymphocytes and macrophages. Clusters of macrophages, so called melanomacrophage centres (MMCs) are present which are involved in immune

memory. Further, the head kidney is responsible for phagocytosis, antigen processing, and production of IgM (Reyes-Cerpa *et al.* 2013). During the early life stage, the entire kidney is responsible for the effective immune response. With maturation of the fish this changes and the anterior kidney is focusing on the immune function while the posterior part is responsible for blood filtration and urinary function (Press and Evensen 1999; Tort *et al.* 2003; Uribe *et al.* 2011).

The development of T Lymphocytes is based in the thymus. These cells are involved in allograft rejection, stimulation of phagocytosis and antibody production by B cells (Press and Evensen 1999; Tort *et al.* 2003; Alvarez-Pellitero 2008; Uribe *et al.* 2011; Reyes-Cerpa *et al.* 2013).

The spleen is the predominant site for phagocytosis and deposition of cells, which are considered to be too old or infected. Furthermore, it has hematopoietic functions similar to the functions of lymph nodes in mammals (Press and Evensen 1999; Ostrander 2000; Uribe *et al.* 2011; Reyes-Cerpa *et al.* 2013).

The mucosa-associated lymphoid tissue in general includes gut, skin and gills. Especially the gut-associated lymphoid tissue (GALT) is highly developed to distinguish between pathogenic and symbiotic bacteria, which is important for the prevalence of diseases (Pérez *et al.* 2010). Although the GALT in teleost fish lacks specialized structures like Peyer's patches in mammals, immune response of the intestinal mucosa is assured by the presence of lymphocytes, plasma cells, granulocytes, and macrophages in the epithelial cells or disseminated throughout the lamina propria (Press and Evensen 1999; Pérez *et al.* 2010).

Innate and adaptive immune response are important for the survival and the major cell population represented in this defence response include lymphocytes, monocytes, macrophages, granulocytes and thrombocytes, mast cells, non-specific cytotoxic cells and dendritic cells (Ellis 1977; Press and Evensen 1999; Alvarez-Pellitero 2008). Comprehensive reviews on fish cytokines and immune response have

been published by Reyes-Cerpa *et al*. (2012) and Zhu *et al.* (2013). Fig. 1-7 gives an overview of the currently known immune cells and their regulating cytokine network.

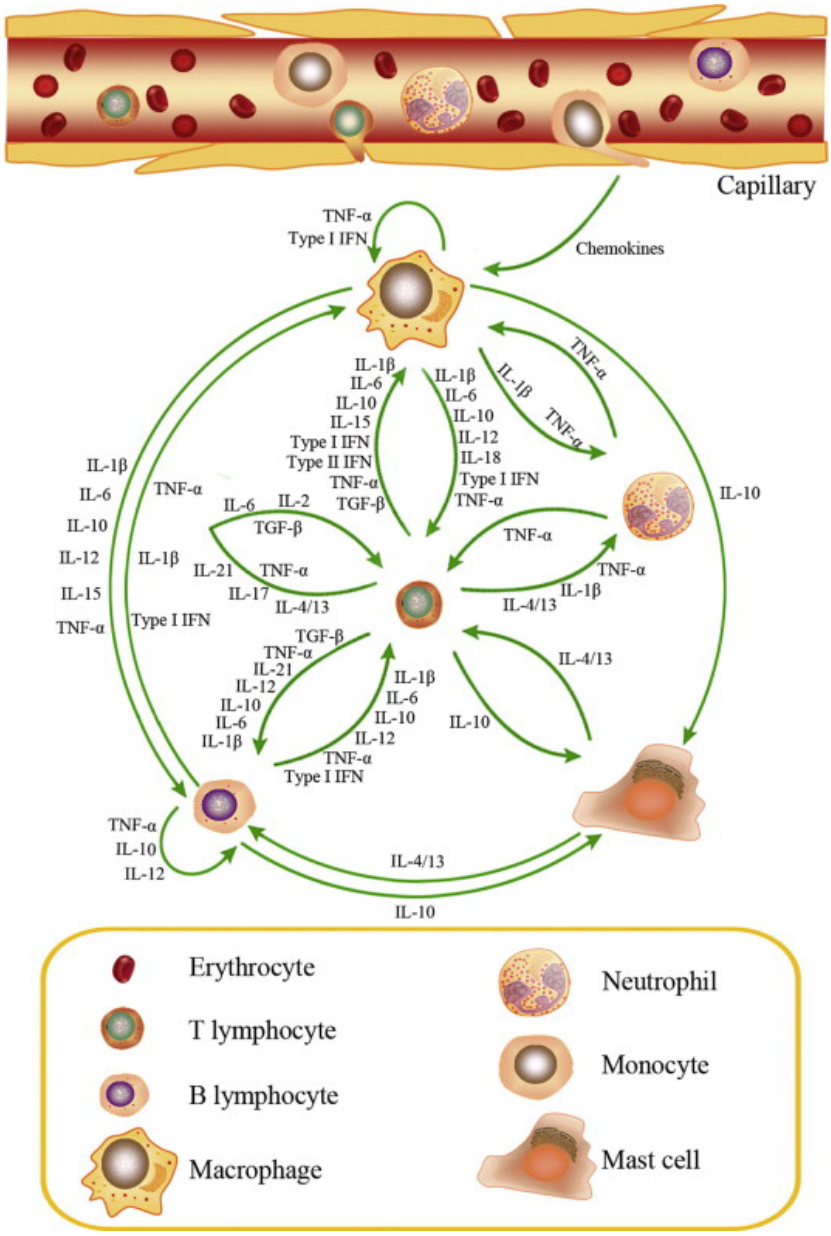

Figure 1-7: Cytokine network of inflammatory cells in fish taken from Zhu *et al*., 2013

1.4.1 Non-specific or innate immune response

During their whole lifecycle, fish live in an open environment and are challenged with pathogens very early during their development. Therefore, innate immune response is essential for fish and they have to depend on it. The innate response can be class-divided into epithelial/ mucosal barriers, humoral parameters and cellular components (Uribe *et al.* 2011). The mucosal and epithelial barriers including the gut, skin and gills provide a barrier to the entry of pathogens and contain a variety of primary defence mechanism responsible for local immune reponses (Press and Evensen 1999; Tort *et al.* 2003; Alvarez-Pellitero 2008; Uribe *et al.* 2011).

Non-specific, humoral factors include growth inhibiting substances like transferrin, anti-proteases and lysins. Lysins are e.g. lysozymes, C-reactive protein (CRP) and most importantly, the complement system which has lytic, pro-inflammatory, chemotactic and opsonic activities thus making a link with non-specific cell-mediated response (Ellis 1999; Uribe *et al.* 2011; Zhu *et al.* 2013).

The non-specific cell-mediated response, also known as non-specific phagocytic response, is primarily executed by neutrophils and macrophages and these are very similar to their human counterparts. They produce reactive intermediates and can be activated by cytokines or the complement system (Ellis 1999; Tort 2011; Uribe *et al.* 2011). Like mammals, fish possess three complement pathway systems; the classical, the alternative and the mannose-binding pathway (Alvarez-Pellitero 2008; Tort 2011; Uribe *et al.* 2011).

Fish and mammals share a lot of similarities in their innate immune system. As well as having the same complement pathways and cell types, a wide range of similar cell receptors have also been discovered, e.g. Toll-like receptors (TLR 2, 4, 5 and 9) and mannose receptors. Also the same cytokines and chemokines have been discovered, e.g. IFNγ, TNFα and Interleukins (Meijer *et al.* 2004; Alvarez-Pellitero 2008; Meeker and Trede 2008; Sullivan and Kim 2008; Takano *et al.* 2011; Uribe *et al.*

2011; Reyes-Cerpa *et al.* 2013; Zhu *et al.* 2013). In addition, similar phagocytosis mechanisms are present in fish, such as inducible nitric oxidase synthase (iNOS) and homologous links to adaptive response, such as major histocompatibility complex (Class I and II) (Alvarez-Pellitero 2008; Meeker and Trede 2008; Uribe *et al.* 2011; Zhu *et al.* 2013). Considering the ontology of the immune system, teleost and elasmobranchs are the most primitive group of vertebrates, which possess a MHC system and T cell receptors (Uribe *et al.* 2011; Zhu *et al.* 2013).

1.4.2 Specific or adaptive immune response

Like mammals, fish have an adaptive immune response. Humoral specific components are anti-adhesins, anti-toxins and anti-invasins which activate the classical pathway of the complement system (Ellis 1999).

Specific cell-mediated immunity has been discovered in fish including T-lymphocytes and B-lymphocytes, which are analogous to the mammalian T helper cells (Th cells or $CD4^+$ cells) and cytotoxic T-cells ($CD8^+$). Additional to the Th1 and Th2 cells, Th17 has recently been discovered in fish (Alvarez-Pellitero 2008; Meeker and Trede 2008; Uribe *et al.* 2011; Reyes-Cerpa *et al.* 2013; Zhu *et al.* 2013). Furthermore, cytokines like IL- 4, 10, 12, 15, 18 and IFNγ as well as TGFβ have been discovered in fish which are important in the adaptive response (Uribe *et al.* 2011; Zhu *et al.* 2013).

Besides T cells, B cells have also been described in fish and are characterized by the expression of B cell receptor, a surface immunoglobulin receptor (sIG). Fish B cells, like those of mammals, have been demonstrated to show Ig H-chain re-arrangement and allelic exclusion. Various immunoglobulin classes have been discovered in fish, e.g. IgM, IgD, IgT and IgZ (Alvarez-Pellitero 2008; Uribe *et al.* 2011; Reyes-Cerpa *et al.* 2013; Zhu *et al.* 2013), but not IgG as found in mammals. Additionally, recent findings suggest that certain B cells which have high affinity receptors might be selected as memory B cells (Uribe *et al.* 2011).

1.4.3 Factors influencing immune response

Several factors have an impact on the fish immune response and can either lead to a reduction or an improvement of the immunological capability of fish. One of the major influencing factors is the environment. Changes in temperature, photoperiod or seasonality have been shown to modify the immune response significantly (Corbel 1975; Schreck *et al.* 2001; Angeles Esteban *et al.* 2006; Magnadóttir 2006; Bowden *et al.* 2007).

Another compromising factor is stress. Stress in fish can have various sources starting with environmental factors like water quality or pollution, over stocking density, ranking order and breeding cycles to husbandry factors like handling and transport (Schreck *et al.* 2001; Barton *et al.* 2002; Magnadóttir 2006; Ramsay *et al.* 2006; 2009a; Tort 2011).

Nutrition also plays an important part in the immune response. Food quality and quantity as well as nutritional availability can influence the immune response in both ways, positive and negative. Nowadays, the use of immunostimulants like beta-glucans in fish food to improve the immunological capability in fish is a major target in aquaculture research (Lall 2000; Magnadóttir 2006; Craig and Helfrich 2009; Kiron 2012).

Besides these external factors, the fish itself is an important varying factor. Changes in age, size and weight influence the immune response. Furthermore, differences can be identified between species, strains and even individuals. The genetics of fish is also an important factor for the variation that is seen between individuals with regard to differences in their innate resistance and acquired immunity (Torroba and Zapata 2003; Tort *et al.* 2003).

The type of infectious agent is also important and the immune response that is elicited by the fish differs between parasites, bacteria or viruses. Furthermore, the

strain and virulence of the pathogen, as well as level of exposure, can influence the immune response (Le Morvan *et al.* 1998; Tort *et al.* 2003; Alvarez-Pellitero 2008).

In most instances, one or more of the factors outlined above are interrelated and have a negative effect on the fish but a positive effect on the pathogen, thus resulting in infection and disease.

1.5 *Zebrafish as a model organism*

1.5.1 Advantages and disadvantages of zebrafish as a model

Although the zebrafish has been used in the laboratory to study embryogenesis for the past 100 years, their potential was only fully discovered in the recent three decades (Meeker and Trede 2008; Sullivan and Kim 2008; Novoa and Figueras 2012). Nowadays modern research is nearly unthinkable without this organism.

The zebrafish, as a model, features numerous advantages compared to mammalian models. The immune system of the zebrafish is similar to that of higher vertebrates in many aspects (Meijer *et al.* 2004; Lieschke and Currie 2007; Novoa and Figueras 2012), containing a fully developed innate and adaptive immune response (van der Sar *et al.* 2004b; Novoa and Figueras 2012). Cells like macrophages, granulocytes, T and B cells, which are considered to be the hallmark of human immunity are present in zebrafish as well as homologues of determinants of innate and adaptive human immunity, including Toll-like receptors, complement components, major histocompatibility class proteins, as well as most cytokines and chemokines (Meijer *et al.* 2004; van der Sar *et al.* 2004b; Meeker and Trede 2008; Sullivan and Kim 2008; Novoa and Figueras 2012). In the class of vertebrates with a completely developed immune system, zebrafish, with a body size of about 5 cm are one of the smallest (Meeker and Trede 2008; Novoa and Figueras 2012).

Due to that small size, zebrafish can be kept in high numbers, requiring only a small space in laboratories compared to other vertebrate model animals (Novoa and

Figueras 2012). Furthermore, zebrafish are a fecund species. A couple of zebrafish can produce 200 – 300 progeny in a week (Meeker and Trede 2008; Novoa and Figueras 2012). Combined with a rapid development, most organs are fully developed five days after fertilization, zebrafish are ideal for chemical screenings. An additional advantage of the zebrafish is their genetically tractability. The entire zebrafish genome has been sequenced (http://www.sanger.ac.uk/Projects/D_rerio/) and is being assembled (http://danio.mgh.harvard.edu/markers/ssr.html/) (van der Sar *et al.* 2004b; Meeker and Trede 2008). Thus, large numbers of genetic markers have been mapped. Furthermore, a variety of mutants has been created to study the influence of specific immune components by knocking them out (Trede *et al.* 2004; van der Sar *et al.* 2004b; Meeker and Trede 2008; Novoa and Figueras 2012). Additionally, other molecular tools like microarrays and deep-sequencing are available for zebrafish. Another useful characteristic of zebrafish is their transparency during embryo and larval stages, which inspired the creation of a transparent mutant called Casper fish at the Children's Hospital Boston (Trede *et al.* 2004; Lieschke and Currie 2007; White *et al.* 2008b; Novoa and Figueras 2012). The Casper zebrafish allows the observation of disease spreading beyond the larval stage and provides the opportunity to investigate the internal anatomy in real-time (White *et al.* 2008b).

Besides all these advantages, the zebrafish model also has some negative aspects. Fish are oviparous, so studies about the vertebrate maternal and in-utero development are not performable with this model. Additionally, although there is much knowledge on the development of the zebrafish, there is a lack of knowledge about their behaviour (Novoa and Figueras 2012).

1.5.2 Existing zebrafish models

1.5.2.1 For fish immune system and disease

Danio rerio as a model organism is used for investigating fish diseases, fish development and the fish immune system (Traver *et al.* 2003; Trede *et al.* 2004; Meijer and Spaink 2011). Already established disease models in this species include those for infectious hematopoietic necrosis (IHN) (LaPatra *et al.* 2000; Wang *et al.* 2006), infectious pancreatic necrosis (IPN*)* (LaPatra *et al.* 2000), spring viraemia of carp (SVC) (Sanders *et al.* 2003; Wang *et al.* 2006; López-Muñoz *et al.* 2010), viral haemorrhagic septicaemia (VHS) (Novoa *et al.* 2006), infectious spleen and kidney necrosis (ISKNV) (Xu *et al.* 2008) and infections resulting from nervous necrosis virus (NNV) (Lu *et al.* 2008). Furthermore, a wide range of bacterial disease models have been developed, e. g. for *Aeromonas hydrophilia* (Rodriguez *et al.* 2008; 2009), *Aeromonas salmonicida* (Lin *et al.* 2007), *M. marinum* (Ramakrishnan *et al.* 1997; Talaat *et al.* 1998; 1999; Cosma *et al.* 2003; Prouty *et al.* 2003; Decostere *et al.* 2004; van der Sar *et al.* 2004a; Broussard and Ennis 2007; Clay *et al.* 2007; Lesley and Ramakrishnan 2008; Meeker and Trede 2008; Sullivan and Kim 2008; Tobin and Ramakrishnan 2008; Parikka *et al.* 2012) and *Streptococcus iniae* (Miller and Neely 2004; 2005; Roca *et al.* 2008; López-Muñoz *et al.* 2009; Phelps *et al.* 2009). Table 1-1 represents an overview of existing zebrafish models for fish, mammal and human diseases.

Zebrafish are also used to investigate the effect of different diets or the effect of husbandry on fish development and immune system (Aleström *et al.* 2006; Dahm and Geisler 2006; Ramsay *et al.* 2006; Ulloa *et al.* 2011).

1.5.2.2 For human disease

The zebrafish model is also used in numerous fields of human research, e.g. developmental biology, oncology, toxicology, reproductive studies, teratology,

genetics, neurobiology, environmental sciences, stem cell and regenerative medicine and evolutionary theory.

Existing models of bacterial diseases include *Francisella* spp (Vojtech *et al.* 2009), the tuberculosis-relative *M. marinum* (Ramakrishnan *et al.* 1997; Talaat *et al.* 1998; Cosma *et al.* 2003; Prouty *et al.* 2003; Decostere *et al.* 2004; van der Sar *et al.* 2004a; Meijer *et al.* 2005; Broussard and Ennis 2007; Clay *et al.* 2007; Lesley and Ramakrishnan 2008; Meeker and Trede 2008; Sullivan and Kim 2008; Tobin and Ramakrishnan 2008; Hegedűs *et al.* 2009; Parikka *et al.* 2012), *Salmonella typhimurium* (van der Sar *et al.* 2003; 2006; Stockhammer *et al.* 2009; 2010; Ordas *et al.* 2011), *Staphylococcus aureus* (Lin *et al.* 2007; Prajsnar *et al.* 2008), *Streptococcus iniae* (Miller and Neely 2004; 2005; Roca *et al.* 2008; López-Muñoz *et al.* 2009; Phelps *et al.* 2009), *Streptococcus suis* (Wu *et al.* 2010) and *Vibrio cholera* (Saslowsky *et al.* 2010; Runft *et al.* 2013). Besides bacterial diseases, fungal pathogens like *Candida albicans* (Chao *et al.* 2010) and viruses like herpes simplex virus (Burgos *et al.* 2008) have been investigated by using the zebrafish model. Examples of existing zebrafish models are summarized in Table 1-1.

The zebrafish is also used to model carcinogenic diseases like leukaemia and other diseases like Parkinson's, Alzheimer's (Langenau *et al.* 2003; White *et al.* 2008b; Mione and Trede 2010; Mizgirev and Revskoy 2010; Newman *et al.* 2011; Blandini and Armentero 2012). In addition the zebrafish is being used in cardiovascular research as a model for blood clotting, blood vessel development, heart failure, congenital heart and kidney disease (Chico *et al.* 2008; Chan and Mably 2010). Furthermore, the zebrafish model has been used to examine toxicity effects, e.g. nicotine or drugs (Petzold *et al.* 2009; D'amico *et al.* 2011; Haldi *et al.* 2012).

Table 1-1: List of published zebrafish model for human and fish disease including route of infection, stage of development of the zebrafish (L larvae, E embryo, A adult) summarized and adapted from van der Sar *et al.* (2004b), Meeker and Trede (2008), Sullivan and Kim (2008) and Meijer and Spaink (2011)

Pathogen	Route of infection	Stage of development	Reference
Bacteria			
Aeromonas hydrophilia	Injection, Immersion	(L) (L) (A) (A)	Rawls *et al.* (2004) Rawls *et al.* (2006) Rodriguez *et al.* (2008) Rodríguez *et al.* (2009)
Aeromonas salmonicida	Immersion	(A)	Lin *et al.* (2007)
Aeromonas veronii	Immersion	(E), (L)	Bates *et al.* (2006)
Bacillus subtilis	Injection	(E) (E)	Herbomel *et al.* (1999) Li *et al.* (2007)
Bacillus sphaericus	Injection	(A)	Grisolia *et al.* (2009)
Bacillus thuringiensis	Injection	(A)	Grisolia *et al.* (2009)
Burkholderia cenocepacia	Injection	(A) (E) (E)	Deng *et al.* (2009) Phennicie *et al.* (2010) Vergunst *et al.* (2010)
Edwardsiella ictaluri	Injection	(A)	Petrie-Hanson *et al.* (2007)
Edwardsiella tarda	Injection, Immersion	(A), (E) (A), (E), (A) (E) (A) (A) (E)	Phelan *et al.* (2005a) Pressley *et al.* (2005) Yazawa *et al.* (2006) Nayak *et al.* (2007) Wang *et al.* (2009) Lin *et al.* (2010) Phennicie *et al.* (2010)
Escherichia coli	Injection, Immersion	(E) (E) (L) (E), (L) (E) (L) (E) (E) (E)	Herbomel *et al.* (1999) van der Sar *et al.* (2003) Rawls *et al.* (2006) Rawls *et al.* (2007) Sieger *et al.* (2009) Szabady *et al.* (2009) Wiles *et al.* (2009) Phennicie *et al.* (2010) Valanne *et al*., 2010
Flavobacterium columnare	Injection, Immersion	(A) (A) (A) (E)	Yazawa *et al.* (2006) Moyer and Hunnicutt (2007) Chang *et al.* (2007) Chang and Nie (2008)
Flavobacterium johnsoniae	Injection, Immersion	(A)	Moyer and Hunnicutt (2007)
Francisella spp	Injection	(A)	Vojtech *et al.* (2009)
Haemophilus influenza	Injection	(E)	Phennicie *et al.* (2010)
Leptospira interrogans	Injection	(E)	Davis *et al.* (2009)
Listeria monocytogenes	Injection	(A) (L)	Menudier *et al.* (1996) Levraud *et al.* (2009)
Listeria spp	Injection	(A)	Menudier *et al.* (1996)
Listonella anguillarum	Injection	(A) (A) (L)	Jackson *et al.* (2007) Rojo *et al.* (2007) Oehlers *et al.* (2010)
Mycobacterium haemophilum	Injection	(A)	Whipps *et al.* (2007b)
Mycobacterium marinum	Injection, Immersion, intubation	(E) (A) (A) (A) - (A)	Davis *et al.* (2002) Prouty *et al.* (2003) Cosma *et al.* (2004) Gao *et al.* (2004) Meijer *et al.* (2004) van der Sar *et al.* (2004a)

		(A), (E)	Volkman *et al.* (2004)
		(A)	Meijer *et al.* (2005)
		(A)	Cosma *et al.* (2006)
		(A)	Gao *et al.* (2006)
		(A)	Swaim *et al.* (2006)
		(A)	Broussard and Ennis (2007)
		(E)	Clay *et al.* (2007)
		(A)	Harriff *et al.* (2007)
		(E)	Clay *et al.* (2008)
		(E)	Davis and Ramakrishnan (2009)
		(E)	
		(A)	Hegedűs *et al.* (2009)
		(L)	van der Sar *et al.* (2009)
		(E)	Tobin *et al.* (2010)
Mycobacterium spp.	Injection	(A)	Kent *et al.* (2004)
		(A)	Watral and Kent (2007)
Mycobacterium peregrinum	Immersion, intubation	(A)	Harriff *et al.* (2007)
Pseudomonas aeruginosa	Injection, Immersion	(L)	Rawls *et al.* (2004)
		(L)	Rawls *et al.* (2006)
		(E), (L)	Rawls *et al.* (2007)
		(E)	Brannon *et al.* (2009)
		(E)	Clatworthy *et al.* (2009)
		(E)	Llamas *et al.* (2009)
		(E)	Vasil *et al.* (2009)
		(E)	Phennicie *et al.* (2010)
Pseudomonas flourescens	Immersion	(E), (L)	Bates *et al.* (2006)
Salmonella arizonae	Injection	(E)	Davis *et al.* (2002)
Salmonella typhimurium	Injection	(E)	van der Sar *et al.* (2003)
		(E)	van der Sar *et al.* (2006)
		(E)	Li *et al.* (2007)
		(E)	Stockhammer *et al.* (2009)
		(L)	Flores *et al.* (2010)
		(E)	Ordas *et al.* (2011)
		(E)	Stockhammer *et al.* (2010)
		(E)	Zakrzewska *et al.* (2010)
Staphylococcus aureus	Injection	(A)	Lin *et al.* (2007)
		(E)	Prajsnar *et al.* (2008)
		(A)	Kao *et al.* (2010)
		(E)	Phennicie *et al.* (2010)
Streptococcus agalactiae	Injection	(A)	Hsieh *et al.* (2010)
		(A)	Peng *et al.* (2010)
Streptococcus iniae	Injection, in vitro	(A)	Neely *et al.* (2002)
		(A)	Miller and Neely (2004)
		(A)	Miller and Neely (2005)
		(A)	Lowe *et al.* (2007)
		(A)	Locke *et al.* (2008)
		(A), (C)	Roca *et al.* (2008)
		(A), (C)	López-Muñoz *et al.* (2009)
		(A)	Phelps *et al.* (2009)
Streptococcus pyogenes	Injection	(A)	Neely *et al.* (2002)
		(A)	Brenot *et al.* (2004)
		(A)	Miller and Neely (2004)
		(A)	Bates *et al.* (2006)
		(A)	Cho and Caparon (2005)
		(A)	Montañez *et al.* (2005)
		(A)	Phelps and Neely (2007)
		(E)	Fisher *et al.* (2008)
		(A)	Rosch *et al.* (2008)
		(A)	Kizy and Neely (2009)

		(A), (L) (A)	Lin *et al.* (2009a) Phelps *et al.* (2009)
Streptococcus suis	Injection	(A) (A)	Wu *et al.* (2008) Wu *et al.* (2010) Zhang *et al.* (2010)
Vibrio anguillarum	Immersion	(E)	O'Toole *et al.* (2004)
Vibrio cholera	Immersion	(E)	Saslowsky *et al.* (2010)
Vibrio vulnificus	Injection	(A) (A)	Hsieh *et al.* (2010) Peng *et al.* (2010)
Yersinia ruckeri	Injection	(E)	Sieger *et al.* (2009)
Virus			
Herpes simplex virus type 1 (HSV-1)	Injection	-	Burgos *et al.* (2008)
Infectious hematopoietic necrosis virus (IHNV)	Injection, in vitro	(A) (C)	LaPatra *et al.* (2000) Wang *et al.* (2006)
Infectious pancreatic necrosis virus (IPNV)	Injection, in vitro	(A) (C)	LaPatra *et al.* (2000) Garner *et al.* (2003)
Infectious spleen and kidney necrosis virus (ISKNV)	Injection, in vitro	(A)	Xu *et al.* (2008)
Nervous necrosis virus (NNV)	Injection	(L), (A)	Lu *et al.* (2008)
Snakehead rhabdovirus (SHRV)	Injection, Immersion, in vitro	(C) (A) (L) (E), (L), (A) (E)	Altmann *et al.* (2003) Alonso *et al.* (2004) Hermann and Kim (2005) Phelan *et al.* (2005b) Nayak *et al.* (2007)
Spring viraemia of carp virus (SVCV)	Injection, Immersion, in vitro	(A) (C) (A), (C)	Sanders *et al.* (2003) Wang *et al.* (2006) Roca *et al.* (2008)
Viral haemorrhagic septicaemia virus (VHSV)	Injection	(A)	Novoa *et al.* (2006)
Fungi			
Candida albicans		(E)	Chao *et al.* (2010)

1.5.3 Zebrafish –*M. marinum* model

Mycobacterial infection is one of the best-described infectious models in zebrafish which is the natural host of *M. marinum* (Clay *et al.* 2007; Meeker and Trede 2008; Sullivan and Kim 2008; Ramakrishnan 2013), the causative agent of fish tuberculosis (Mycobacteriosis). *M. marinum* is biosafety level-2 (BSL-2) and its generation time is 4 h compared to *M. tuberculosis* with a generation time of 20 h and a BSL-3 status (Broussard and Ennis 2007; Tobin and Ramakrishnan 2008). Besides this, the near relation (99, 4%) based on a 16 S – rRNA analysis to *M. tuberculosis* qualifies *M. marinum* as an ideal alternative (Talaat *et al.* 1998; Cosma *et al.* 2003; van der Sar *et al.* 2004a; Hagedorn and Soldati 2009).

M. marinum replicates like *M. tuberculosis* in host macrophages and initiates a chronic granulomatous infection using shared virulence determinants (Lesley and Ramakrishnan 2008; Hagedorn and Soldati 2009; Ramakrishnan 2013). The typical lesion which can be seen following histopathology examination is the granuloma, which can be present in any internal organ. The histopathology of the formed granuloma in fish tuberculosis is similar to the histopathology seen in human tuberculosis (Ramakrishnan *et al.* 1997; Talaat *et al.* 1998; Prouty *et al.* 2003; Decostere *et al.* 2004; Meeker and Trede 2008; Sullivan and Kim 2008; Berg and Ramakrishnan 2012; Parikka *et al.* 2012; Ramakrishnan 2013).

M. marinum granulomas also exhibit the essential core of epithelioid cells and often with the zippered appearance of cell membranes (Bouley *et al.* 2001; Ramakrishnan 2004; Berg and Ramakrishnan 2012). Similar to *M. tuberculosis*, *M. marinum* produces caseating or non-caseating granulomas depending on the infected host organism. Both caseating and non-caseating granulomas can be found in certain toads, goldfish, zebrafish, and humans (Talaat *et al.* 1998; Bouley *et al.* 2001; Ramakrishnan 2004; Swaim *et al.* 2006; Parikka *et al.* 2012). Especially in zebrafish, caseating and non-caseating granulomas can be found within the same individual.

The similar ethiopathology of *M. marinum* infections in zebrafish in combination with the advantages of the zebrafish as a vertebrate model organism provides the optimal requirements to study the interactions between *M. marinum* and the zebrafish as a model for human tuberculosis. A variety of research groups are already working on different approaches to gain further insights into tuberculosis by using this model (Ramakrishnan *et al.* 1997; Davis *et al.* 2002; Prouty *et al.* 2003; Cosma *et al.* 2004; Gao *et al.* 2004; Meijer *et al.* 2004; van der Sar *et al.* 2004a; Volkman *et al.* 2004; Meijer *et al.* 2005; Cosma *et al.* 2006; Gao *et al.* 2006; Swaim *et al.* 2006; Broussard and Ennis 2007; Clay *et al.* 2007; Harriff *et al.* 2007; Clay *et al.* 2008; Davis and Ramakrishnan 2009; Hegedűs *et al.* 2009; van der Sar *et al.* 2009; Tobin *et al.* 2010; Volkman *et al.* 2010; Parikka *et al.* 2012; Ramakrishnan 2013).

Most of these studies are focused on the innate immune response using juvenile individuals like embryos or larvae as model organisms in the absence of the adaptive immune response (Davis *et al.* 2002; Volkman *et al.* 2004; Clay *et al.* 2007; Clay *et al.* 2008; Davis and Ramakrishnan 2009; Hegedűs *et al.* 2009; Tobin *et al.* 2010; Volkman *et al.* 2010). Another main topic is the formation and dissemination of granuloma in acute and chronic disease stages (Cosma *et al.* 2003; Swaim *et al.* 2006; Broussard and Ennis 2007; Meijer *et al.* 2008; Davis and Ramakrishnan 2009). The immune response of adult zebrafish to *M. marinum* infections is not well described yet. Recently, a latent tuberculosis model using adult zebrafish and *M. marinum* was published (Parikka *et al.* 2012) leading the way for further investigations. Due to the importance of the adaptive response in human *M. tuberculosis* infection, investigation of the adaptive immune system of *M. marinum* infected zebrafish may gain new insights into the complex host-mycobacterium-relationship.

1.6 *Aims and Objectives*

The main aim of this thesis was to investigate the disease progression and mechanisms involved in *M. marinum* infection in adult zebrafish with respect to pathogenesis and host defence mechanisms under active, latent and re-activated stage of the disease. Previous studies focusing on the fish model so far have investigated embryonic and larval immune response.

The objectives to achieve this were;

(1) To study early macrophage aggregation and granuloma formation during disease progression in adult zebrafish accompanying *M. marinum* infection and compare this with *M. tuberculosis* granuloma, the hallmark of this human disease as well as to mycobacterial granuloma found in other fish species (Chapter 3).

(2) To determine the virulence and infectivity of two *M. marinum* strains in adult zebrafish, i.e. NCIMB 1297 and NCIMB 1298 (Chapter 3 and 4).

(3) To develop methods for the isolation, *in vitro* culture and infection of macrophages from individual zebrafish followed by RNA extraction and establishment of a real-time RT-PCR procedure to characterise the effect of *M. marinum* infection on expression of immune related genes in zebrafish macrophages (Chapter 4).

(4) To investigate innate immune response in the kidney of adult zebrafish to *M. marinum* infections by focusing on pro-inflammatory cytokines such as Interleukin 1 beta, Tumor necrosis factor alpha, Interleukin 12 and Interferon gamma which are proven to be relevant in human tuberculosis infections (Chapter 4).

(5) To assess the host response to long term and reactivated *M. marinum* infection in the kidney of adult zebrafish using transcriptomics and qRT-PCR to evaluate similarities to human and mammalian reactivation studies (Chapter 5).

2 Materials and Methods

2.1 *Zebrafish husbandry and breeding*

2.1.1 Zebrafish husbandry

Zebrafish were maintained in a stand-alone zebrafish recirculation housing system (Aquaneering Inc.) (Fig. 2-1) in the tropical aquarium facility of the Institute of Aquaculture, University of Stirling. The holding capacity of this system was approximately 10 adult zebrafish per litre, although younger and smaller fish were stocked at higher densities. A filter and UV bypass provided the system with good water quality. Water temperature was maintained at 27°C with a flow rate of 150 L min^{-1}. The fish were feed once a day with fry food SDS 400 (Special Diets Services, www.sdsdiets.com).

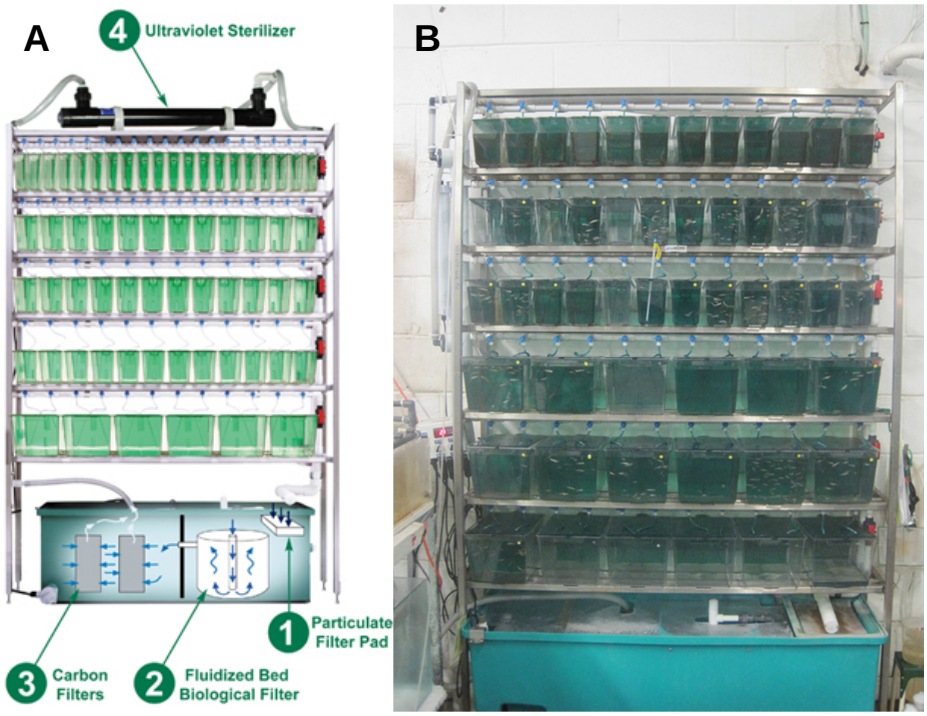

Figure 2-1: Zebrafish Aquatic Housing Systems, (A) Stand Alone Racks, Self-Contained Single-Sided Rack (5-Shelf), Aquaneering Inc.; (B) 6-shelf set-up facility in the Tropical Aquarium, Institute of Aquaculture.

The breeding of zebrafish was performed in a separate aquarium tank next to the zebrafish system, to allow disinfection of the eggs by bleaching in 0.05% sodium hypochlorite (Westerfield 2007) for 5 min before introducing them into the zebrafish system. Breeding was carried out over marbles and synchronised with the onset of the light cycle as described by Westerfield (2007).

Adult zebrafish used for the infection experiments in this thesis were transferred to a separate aquarium, the Aquatic Research Facility (ARF) and acclimated in 10 L tanks for 1 - 2 days before the start of an experiment. The experimental tanks were connected to the aquarium flow-through system. Effluent from the system was treated with ozone and temperature was constantly monitored. Zebrafish were handled according to Home Office regulations.

2.1.2 Maintenance of a mycobacteria free system

To maintain a mycobacteria-free zebrafish stock, water from the zebrafish housing system in the tropical aquarium was checked routinely using a mycobacteria-specific polymerase chain reaction (PCR). Water samples were collected in 50 mL falcon tubes from individual tanks and from the filter systems. These were centrifuged at high speed (15000 x g) for 15 min in a Sigma 4K15 laboratory centrifuge (Sigma-Aldrich Company Ltd, Dorset, UK) to concentrate any mycobacteria into a pellet for the extraction of DNA and subsequent analysis by PCR.

DNA was routinely isolated from these eggs using a Nucleospin® Tissue kit from Macherey-Nagel (Fisher Scientific, Loughborough, UK) to extract genomic DNA from tissue following the user's manual (2.5.1.) and subsequently screened by PCR (2.5.6.). The primers used for screening the system were the genus-specific TB11/12, which target the 65kb heat-shock-protein gene (Telenti *et al.* 1993).

Furthermore, the zebrafish in the system were also screened to check for any underlying pre-infection with mycobacteria. The breeding and ongrowing of new fish

was performed outside of the system, and the resulting *Mycobacterium*-negative eggs were bleached before adding them into the system.

2.1.3 *Mycobacterium* Infection procedure

Zebrafish utilised for the infection experiments were anaesthetized with benzocaine prior to injection. Briefly, the fish were transferred into a small plastic bucket filled with warm water (~ 27°C) and an appropriate volume of 10% benzocaine (Sigma-Aldrich) was added until the desired state of unconsciousness was reached. The starting concentration of the anaesthesia was 0.5 mL benzocaine in 500 mL water which was slowly increased until fish lost consciousness. The anaesthetised fish were carefully taken out of the water and injected with the appropriate inoculums into the coelomic cavity. The injection was performed using a BD Ultra-Fine™ 3/10 cc 30 G needle insulin syringe. After infection, the fish were transferred to another small container filled with oxygenated warm water to let them recover. After the fish regained consciousness, they were checked for any signs of damage due to the injection,and then they were transferred back into the tanks connected to the flow-through-system located in the ARF.

2.2 *Mycobacterium marinum*

2.2.1 Bacterial culture

Mycobacterium marinum strains NCIMB 1297 (ATCC 297) and 1298 were grown on Sauton's modified medium (Tsukamura 1965). Short-term cultivation was performed by growing the cultures on agar plates at 28°C; long-term cultivation was performed by growth on agar slopes at 22°C.

2.2.2 Preparation of mycobacteria for infection of zebrafish

The infection inoculum was prepared on the day of use as follows: Briefly, 1 - 2 colonies were taken with a sterile loop from the mycobacteria plates and transferred to

a 15 mL falcon tube where they were re-suspended in 3ml sterile 1x phosphate buffered saline (PBS), pH 7.2. The mixture was centrifuged for 5 min at 3000 x g at 22°C in a Sigma 4K15 laboratory centrifuge (Sigma-Aldrich) to pellet the bacteria. The supernatant was discarded and the pellet re-suspended in 3 mL sterile PBS containing 0.2% Tween 20 (Thermo Scientific, Fisher Scientific Limited, Loughborough, UK). The mixture was centrifuged an additional time at 200 x g for 5 min to separate the bacteria (Dionne Lab 2013). The supernatant, which contained single bacteria and some cohered, was transferred to a new micro-centrifugation tube and the optical density was measured at 610 nm. An optical density of 0.6 equated to 10^8 colony forming units (CFU) (personal correspondence Taryn Fletcher, Imperial College London, UK). The desired bacterial load was adjusted by dilution series.

The final volume used in all experiments was 20 µL of 10^4 cfu mL^{-1} which resulted in 2.5 x 10^2 cfu per fish *in vivo*.

2.3 *General sampling methods*

2.3.1 Sacrifice of zebrafish

Zebrafish were sacrificed according to the Animals (Scientific Procedures) Act 1986 declared in the UK Home Office regulations regarding working with laboratory animals. All zebrafish were overdosed with 10 % benzocaine (w/v) (Sigma-Aldrich) and killed following Home Office regulations prior to sampling. Samples used for histology only were fixed in 10% neutral buffered formalin (NBF) and stored until processing.

2.3.2 Organ dissection

Organ dissection was difficult due to the size of the fish and therefore zebrafish were transferred onto a paraffin wax-filled Petri dish and transfixed with needles (Fig. 2-2a) prior to dissection. The abdomen was opened and the liver and gut removed. The spleen, which was clearly visible, was sampled and the remaining internal organs

were also sampled or removed, depending on the experiment. Finally, the kidney was collected (Fig. 2-2a-c).

The organs were either homogenized in 300 µL TriReagent (Sigma-Aldrich) with a BD Micro-Fine Plus 1 mL insulin syringe or covered with RNAlater (Sigma-Aldrich) and stored at - 70°C until further processing.

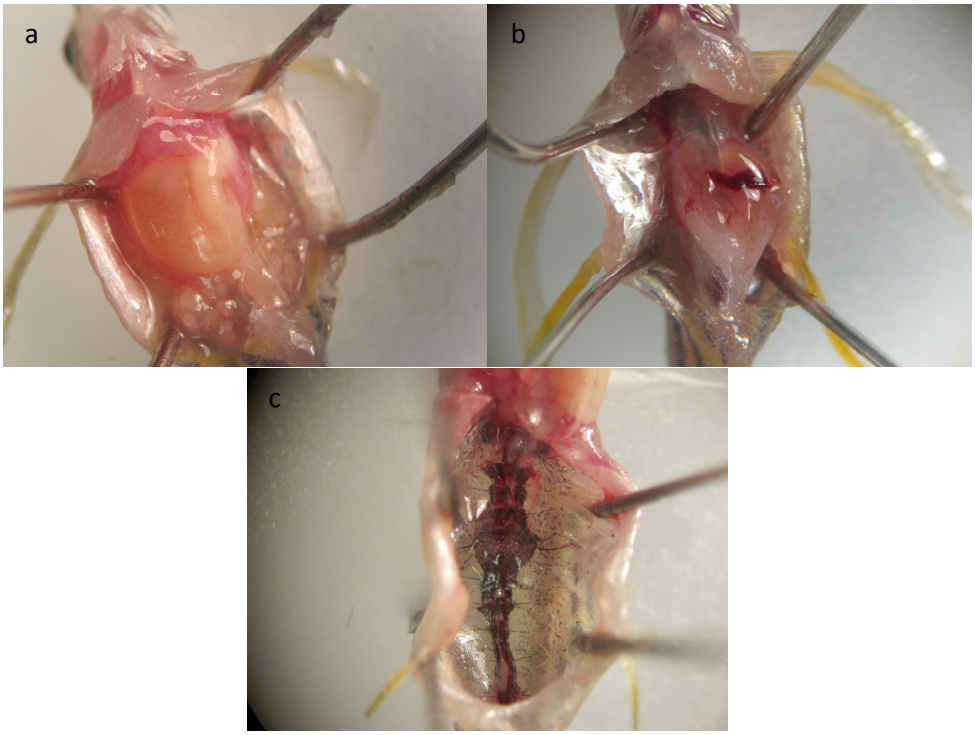

Figure 2-2: Sacrificed zebrafish (a) transfixed with needles on a paraffin wax filled petri dish prior to organ sampling, (b) after removal of gut and liver and close-up view of the exposed spleen and (c) with internal organs removed and close-up view of the exposed kidney prior to its removal.

2.4 *General histological methods*

2.4.1 Processing, embedding and sectioning

The zebrafish samples fixed in 10% NBF were processed in a Shandon Citadel 2000 tissue processor. Prior to processing the tissues were placed in cassettes following the Institute of Aquaculture-Histopathology Lab protocol for cassetting samples. The tail and fins of the zebrafish were removed if possible to allow a better diffusion of the reagents. The cassettes were subsequently placed into the processing

machine, moved consecutively through a series of dehydrating solvents, then finally impregnated with wax. The chronology of the tissue processing in its single steps is described in Table 2-1.

After processing, the cassettes containing tissues were placed in the cassette wax bath of a Leica histo-embedder. Tissues were embedded in paraffin wax and then placed on the cold-plate to allow the paraffin wax to harden. Sectioning of the zebrafish was performed with a Leica microtome. The thickness of the sagittal sections was adjusted to 7 µm according to the ZIRC Health Services Zebrafish Disease Manual (Kent *et al.* 2012) (http://zebrafish.org/zirc/health/diseaseManual.php).

Table 2-1: Summary of the tissue processing steps applied to dehydrate and impregnate tissue samples

Step	Reagent	Time (min)
1	50% Methylated spirits	0.30
2	80% Methylated spirits	1.30
3	100% Methylated spirits	1.30
4	100% Methylated spirits	1.30
5	100% Methylated spirits	1.30
6	Absolute Ethanol	1.45
7	Absolute Ethanol	1.30
8	Chloroform	0.50
9	Chloroform	0.50
10	Paraffin wax	1.45
11	Paraffin wax	1.30
12	Paraffin wax	1.30

2.5 *General molecular methods*

2.5.1 DNA extraction

DNA was isolated from samples using the Nucleospin® Tissue kit from Macherey-Nagel to extract genomic DNA from the sample following the user's manual. Briefly, the samples were pre-lysed by addition of 180 µL T1 buffer and 25 µL proteinase K (Sigma-Aldrich) to each sample. After an overnight incubation at 56 °C, 200 µL B3 buffer was added to the samples and lysis was performed at 70°C for 10 min. After this step, 210 µL ethanol were added to the sample to adjust the DNA binding conditions and the sample was loaded on to a column to bind the DNA. Samples were centrifuged for 1 min at 11000 x g and the flow-through discarded afterwards. Samples were washed twice, once with 500 µL BW buffer and the second time with 600 µL B5 buffer. After each washing step the samples were centrifuged for 1 min at 11000 x g and the flow-through discarded. To dry the silica membrane from any remaining washing buffer, the column was centrifuged for an additional 1 min at 11000 x g. The samples were transferred into new 1.5 mL RNase-free micro-centrifuge tube and the DNA was eluted by adding 100 µL pre-warmed (70°C) BE buffer directly on the membrane. After 1 min incubation at 22°C, the samples were centrifuged one last time for 1 min at 11000 x g. The amount of isolated DNA was quantified with NanoDrop and afterwards stored at - 70°C until further use.

2.5.2 RNA extraction with TriReagent

Tissue samples that had been homogenized in TriReagent as described in Section 2.3.2 were filled up to a volume of 500 µL with TriReagent. Samples stored in RNAlater were removed from it and homogenized in 500 µl TriReagent. After complete homogenization, 5 µg of linear polyacrylamide (LPA) (GenElute, Sigma-Aldrich) and 50 µL of 1-Bromo-3-chloropropane (BCP) (Sigma-Aldrich) were added to the samples and the suspension was mixed for 15 sec. LPA works as a carrier and supports RNA

extraction from small sample sizes. After incubation for 15 min at 22°C, the samples were centrifuged at 12000 x g at 4°C for phase separation. The generated aqueous phase, approximately 200 – 250 µL, was transferred to a new RNase-free 1.5 mL micro-centrifugation tube and the RNA was precipitated by the addition of 250 µL Isopropanol. The solution was mixed by inverting the tube and incubated at -20°C overnight. The samples were subsequently centrifuged at 19000 x g for 30 min at 4°C and the supernatant discarded very carefully. The remaining RNA pellet was washed twice with ice-cold ethanol. The first washing step was performed with 500 µL ice-cold 75% ethanol. The pellet was re-dissolved in the ethanol and centrifuged at 19000 x g for 10 min. The supernatant was discarded and the second washing step was performed in a similar manner to the first one. After removing the supernatant, the RNA pellet was allowed to air dry before re-suspending it in 20 µL RNase-free water. The isolated RNA was kept on ice for 1-2 h or at 4°C overnight to allow the RNA to completely dissolve in the water. The quantity of RNA was determined using the NanoDrop and the quality was verified by gel electrophoresis if possible (Section 2.5.4).

2.5.3 RNA extraction using Qiagen RNeasy Mini Kit

RNA extraction was performed following the manufacturer's protocol for animal tissues. Briefly, tissue samples were disrupted in 300 µL RLT buffer containing 1% β-mercaptoethanol (Sigma-Aldrich) using a BD Ultra-Fine™ 3/10 cc 30 G needle insulin syringe. One volume of 70% ethanol was added immediately to the samples and mixed by pipetting. The lysate was transferred to an RNeasy spin column placed in a 2 mL collection tube and centrifuged for 15 s at ≥ 8000 x g. The supernatant was discarded and 700 µL of Buffer RW1 were added to the RNeasy spin column. The supernatant was discarded again and 500 µL buffer RPE, diluted in 4 volumes of 95-100% ethanol, were added to the column. Following a centrifugation step for 15 s at ≥ 8000 x g and the removal of the supernatant, the column was washed again with

500 µL RPE buffer for 2 min at ≥ 8000 x g. The RNeasy column was placed on a new 2 mL collection tube and centrifuged for 1 min at full speed to remove any remaining buffer solution. After transferring the column into a new 1.5 mL collection tube, 30 µL of pre-warmed water (55°C) were pipetted on the middle of the column membrane and centrifuged for 1 min at ≥ 8000 x g to elute the RNA. The quantity of the RNA was determined using a NanoDrop and the quality was verified by gel electrophoresis if enough RNA was obtained.

2.5.4 Quantification and Qualification of extracted RNA and DNA

To quantify the amount of extracted nucleic acids, 1.5 µL of each sample were applied on a NanoDrop™ 1000 Spectrophotometer. The NanoDrop® software calculated the nucleic acid concentration depending on RNA or DNA input and chosen blank.

The quality of the RNA was determined if enough RNA was obtained by electrophoresis. A minimum amount of 250 ng of each sample was mixed with 10x Reddyrun Gel loading buffer (Thermo Scientific). Samples were loaded on a 1.5% agarose gel and run for 45 min at 80 V (see Section 2.5.8.).

2.5.5 Complementary DNA synthesis

The synthesis of complementary DNA (cDNA) was performed by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK) following the manufacturer's protocol. Briefly, up to 2 µg of total RNA per 20 mL reaction was used. After all components thawed on ice, a 2 X RT master mix was prepared. For each reaction, 2 µL 10 X RT buffer were mixed with 0.8 µL of 25 X dNTP mix, 2 µL 10 X RT Random Primers, 1.0 µL MultiScribe™ Reverse Transcriptase and 3.2 µL Nuclease-free water. Additionally, 1.0 µL RNase Inhibitor (Applied Biosystems) was added to the master mix. 10 µL of the master mix were added to 10 µL of RNA sample and mixed well by pipetting. The tubes were briefly

centrifuged to remove any air bubbles and placed on ice until loading into the thermal cycler. The reverse transcription was performed using the following conditions: 10 min at 25°C, 120 min at 37°C and 5 min at 85°C. Afterwards, the samples were stored at -20°C until further use.

2.5.6 Polymerase Chain Reaction (PCR)

PCR reactions were prepared in RNase-free 0.2 mL thin-wall tubes (AB gene, Thermo Scientific). Equal amounts of the templates, RNA or DNA, were added into 25 µL PCR reaction containing 12.5 µL PCR MasterMix (2x Reddy Mix, Thermo Scientific), RNase-free water and 0.5 µL of each primer. Furthermore, a suitable positive control and a negative control which contained RNase-free water instead of the template were produced for each PCR reaction. The PCR reaction was performed in a Biometra® T Gradient thermocycler. The PCR was carried out using the following thermal profile: 5 min initial denaturation at 94ºC followed by 35 cycles of denaturation at 94ºC, annealing at 57.5ºC, depending on optimal primer annealing temperature, and extension at 72ºC for 1 min and final incubation at 72ºC for 5 min.

2.5.7 Agarose gel electrophoresis

Subsequently, the PCR products were analysed using agarose gel electrophoresis. Briefly, 6 µL of each product were loaded onto a 1.5% agarose gel. The gel was prepared by mixing 1.5% Iberose high specific agarose powder for electrophoresis (Web Scientific, Crewe, UK) with 1x sodium Tris-acetate-EDTA (TAE) buffer. The agarose was dissolved by heating in a microwave without boiling it. Afterwards, the mixture was cooled to approximately 55ºC. Once cool enough, the fluid was poured into a gel tray which was placed into a fume hood, 1.3 µL ethidium bromide (Promega, Southampton, UK) were added with care and compounded with a gel comb. An appropriate comb was inserted to form the wells. The gel was given approximately 30 min to one hour to set. Once the gel had set, the gel together with

the tray was transferred into an electrophoresis chamber. The chamber was filled with 1x TAE and the comb carefully removed without disrupting the chambers.

Six µL of the PCR products were loaded into the wells. To evaluate the size of the products afterwards, a marker mix, containing 1 µL loading dye, 1 µL marker and 10 µL RNase-free water was additionally loaded into one or two of the wells. Electrophoresis was performed at 80 volts for 45 min.

Visualization of the DNA in the gel was enabled under ultraviolet (UV) light and a digital image were taken using the SynGene software (Cambridge, UK).

2.5.8 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed in RNase-free 0.2 mL 96-well plates (THERMO-FAST®, Thermo Scientific). Complementary DNA from total RNA acted as template and each sample was tested in triplicate for each gene in a real-time PCR system (Eppendorf Mastercycler ® ep realplex). Ten microliters of ABSOLUTE™ QPCR SYBR® Mix (Thermo Scientific) were mixed with 0.6 µL of 10 µM forward and reverse primer, 3.8 µL of nuclease free water and 5 µL of template. The 96-well plate was sealed with an ABSOLUTE™ QPCR SEAL (Thermo Scientific) after the reaction mix was loaded into the plate. After mixing the samples by flicking and a brief centrifuge step, the plate was placed into the Mastercycler. The reaction was carried out with the following cycling conditions: After a 15 min enzyme activation step at 95°C, 40 cycles were performed with denaturation at 95°C for 15 s, annealing at 57.5°C - 65°C for 20 s, depending on optimal primer annealing temperature, and extension at 72°C for 30 s. Subsequently, a melting curve was measured after each run.

3 Analysis of granuloma formation in adult zebrafish (*Danio rerio*) infected with *Mycobacterium marinum*

3.1 *Introduction*

The granuloma is the primary pathological lesion that can be detected in fish infected with mycobacteriosis (Jacobs *et al.* 2009; Francis-Floyd 2011). During early stages of piscine mycobacteriosis, granulomas have been predominately observed in kidney, liver and spleen, while at more advanced stages, further organs can also be infected (Swaim *et al.* 2006; Jacobs *et al.* 2009; Parikka *et al.* 2012). In general, the fundamental structure of the piscine granuloma is characterised by a central area of necrosis, which is surrounded by macrophages, epithelioid cells and fibrous connective tissue (Talaat *et al.* 1997; Swaim *et al.* 2006; Gauthier and Rhodes 2009; Jacobs *et al.* 2009; Francis-Floyd 2011). Other features like multinuclear giant cells, caseous necrosis, calcification and fibrosis have also been observed in some fish species infected with mycobacteria (Majeed *et al.* 1981; Talaat *et al.* 1998; Swaim *et al.* 2006; Gauthier and Rhodes 2009), but these are not considered to be general characteristics of the disease. In fish, granulomas generally exhibit few lymphocytes, a feature which fish share with frogs, but which distinguishes them from mammals (Bouley *et al.* 2001; Swaim *et al.* 2006; Tobin and Ramakrishnan 2008).

A range of *Mycobacterium* species have been proven to cause mycobacteriosis in fish and other aquatic animals of which *M. marinum* is the most frequently reported, followed by *M. fortuitum* and *M. chelonae* (Jacobs *et al.* 2009; Francis-Floyd 2011). The susceptibility of fish to mycobacteriosis is highly dependent on fish species, mycobacterial species and the isolate. Different mycobacterial species, strains and strain isolates can lead to the development of a variety of disease outcomes in the same fish species, as well as variation between fish species (Majeed *et al.* 1981;

Talaat *et al.* 1998; Talaat *et al.* 1999; Swaim *et al.* 2006; Gauthier and Rhodes 2009; Jacobs *et al.* 2009; Francis-Floyd 2011; Parikka *et al.* 2012). With regard to the disease outcome between, as well as within, fish species, the actual progression of the mycobacterial disease is hard to generalise.

Some *Mycobacterium* species isolated from fish, such as *M. marinum* and *M. fortuitum*, are zoonotic to humans and cause granulomatous lesions predominately in the skin of hands and other extremities (Panek and Bobo 2006; Francis-Floyd 2011). The granulomas caused by *M. marinum* in humans are similar to the granuloma induced by *M. tuberculosis*.

Human tuberculosis-induced granulomas exhibit a centre of caseous necrosis surrounded by macrophages and epithelioid cells and are defined by a cuff of lymphocytes and fibrotic connective tissue. Calcification and fibrosis can be frequently observed (Basaraba 2008; Barry *et al.* 2009; Reece and Kaufmann 2012). This type of granuloma is the classical tuberculosis granuloma and can be detected in active and latent disease. The bacteria are walled off from the surrounding healthy tissue and can be detected in the macrophages, the necrotic centre or in the fibrotic border area (Basaraba 2008; Barry *et al.* 2009; Reece and Kaufmann 2012). In the late stage of tuberculosis, granulomas can also be found with multi-centric necrosis depending on the severity of the active disease (Swaim *et al.* 2006). Depending on the disease progression and the individual health status of the host, several different types of granuloma can be present.

In theory, granuloma formation starts with early association of macrophages, which accumulate to form a solid or non-necrotic granuloma (Fig. 3-1). The early granuloma contains different types of immune cells including T cells, B cells and neutrophils which are encircled by fibroblasts as the granuloma progresses.

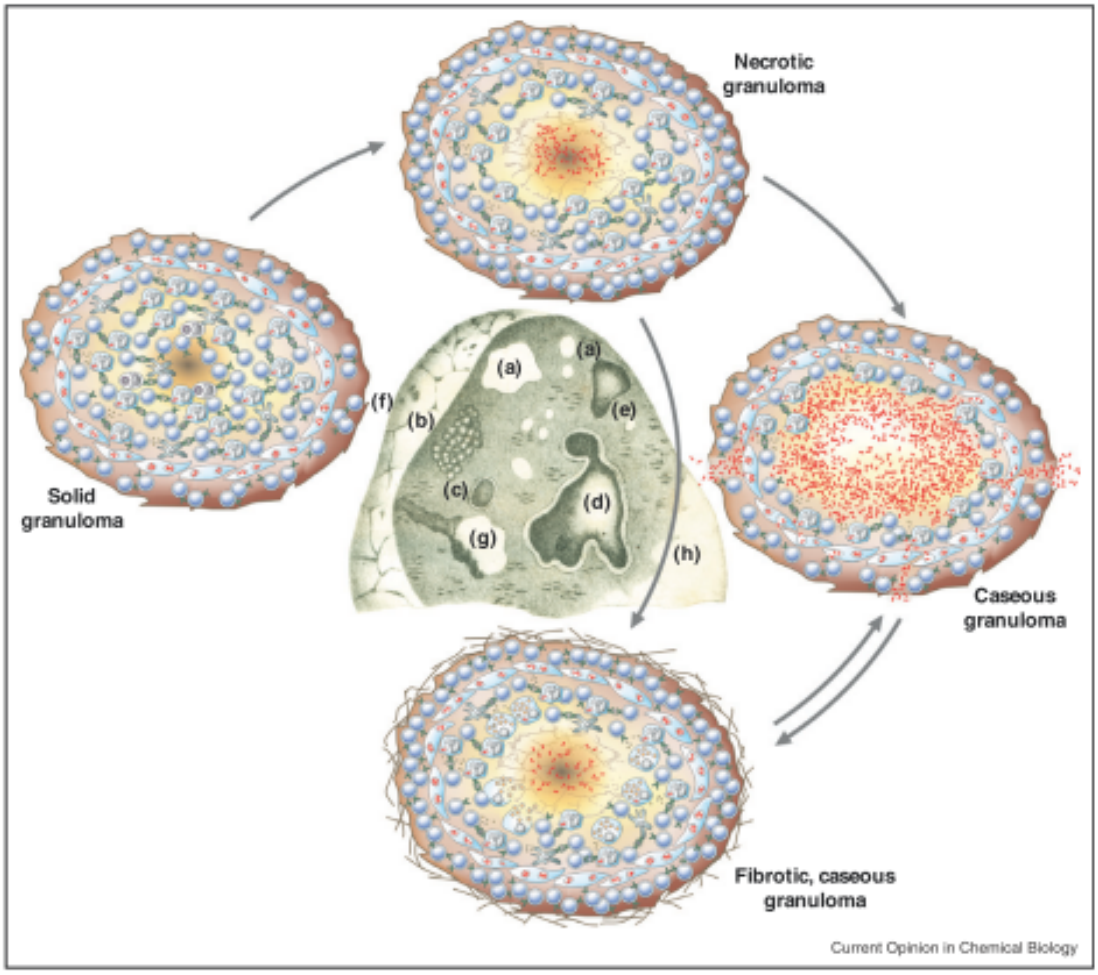

Figure 3-1: Overview of different granuloma phenotypes observed in active tuberculosis in humans taken from Reece and Kaufmann (2012). The figure shows (a-h) granuloma structures illustrated by Bayle about 200 years ago and the current view on granuloma types including solid, necrotic, caseous and fibrotic caseous granuloma as well as their progression from one stage to another.

As the disease stage progresses, the centre of the granuloma can become caseous with necrosing cells, in which granuloma state it is referred to as a caseous or a necrotic caseating granuloma (Fig. 3-1) (Reece and Kaufmann 2012).

Necrotic granulomas are generally believed to develop from non-necrotic ones. The infected phagocytes, mostly macrophages, undergo necrosis which leads to build up of cell debris in the centre of the granuloma. In the later stages, this necrotic centre softens without any cellular outline. From this point it is referred to as caseum or caseating necrosis due to its cheese-like appearance resulting from extensive cell death (Basaraba 2008; Barry *et al.* 2009; Reece and Kaufmann 2012). The caseum

provides an environment in which the bacteria can multiply undisturbed, and liquefaction followed by cavitation of a caseous granuloma is believed to be one possible source of re-activation, although most of the contributing factors are still unclear.

The similarities in the granuloma composition caused by *M. tuberculosis* and *M. marinum* in humans, in addition to the close genetic relationship of the two pathogens, makes *M. marinum* an ideal surrogate model pathogen to investigate tuberculosis. Furthermore, *M. marinum* causes granulomas in fish and frogs, with many of the features observed in human granuloma. This offers the possibility of non-mammalian models to investigate mycobacterial diseases (Broussard and Ennis 2007). Candidates of non-mammalian *M. marinum* models have been narrowed down to goldfish (*Carassius auratus*), leopard frog (*Rana pipiens*), medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) (Ramakrishnan *et al.* 1997; Talaat *et al.* 1998; Bouley *et al.* 2001; Broussard and Ennis 2007). *M. marinum* infection studies have been performed in all four of these candidates revealing that zebrafish and medaka are the most suitable ones. Leopard frogs do not develop necrosis in their granulomas (Ramakrishnan *et al.* 1997; Bouley *et al.* 2001), which is a major disadvantage in modelling human tuberculosis and piscine mycobacteriosis as it is one of the major features in both diseases (Cosma *et al.* 2003; Gauthier and Rhodes 2009; Jacobs *et al.* 2009; Ramakrishnan 2012). In comparison to the goldfish, zebrafish and medaka offer the possibilities of various mutant and transgenic lines and an extensive research data base. Both zebrafish and medaka also possess sequenced genomes, which can provide additional sources of information with respect to the host response to infection. In addition, the zebrafish is known as a natural host to *M. marinum*, whereas natural infections of medaka tend to be caused by *M. fortuitum* and *M. abscessus* (Broussard and Ennis 2007).

The aim of this chapter was to gain a better understanding of granuloma formation caused by *M. marinum* and disease progression in adult zebrafish over short and long-term infection by:

(1) Comparing experimental infection challenges with *M. marinum* isolates NCIMB 1297 and 1298, the latter of which has been previously reported to be non-infective to zebrafish and following subsequent granuloma formation in the host over 14 days following infection.

(2) Investigating the long-term disease progression following *M. marinum* (NCIMB 1297) infection in zebrafish by examining granuloma formation in fish in terms of:

   I. Number of affected organs

   II. Total number of granulomas in affected tissues

   III. Type of granulomas detectable

(3) Determinate if granulomas in zebrafish exhibit signs of caseation, fibrosis and calcification, key features in latent human tuberculosis granulomas.

The results and information gained from this Chapter were used to verify the applicability of the zebrafish-*M. marinum* model in the study of granuloma formation in latent human tuberculosis infection (LTBi).

3.2 *Materials and Methods*

3.2.1 Fish husbandry

Zebrafish husbandry prior to and during the infection experiment has been described in detail in Chapter 2.1.1.

3.2.2 Bacterial strain, culture conditions and inoculum preparation

M. marinum isolates NCIMB 1298 and 1297 were used in this chapter. Culture conditions for each strain and the preparation of the individual inoculums were as described in Chapter 2.2.

3.2.3 Zebrafish infection and experimental outline

3.2.3.1 Infection procedure

Zebrafish were anaesthetised and infected with 20 µL of 10^4 cfu mL^{-1} of each inoculum or sterile phosphate buffered saline (PBS) as described in Chapter 2.1.2.

3.2.3.2 Experiment set up

A total of 136 fish were investigated in two experiments. The first experiment comprised 40 fish injected with either sterile PBS (n = 16), *M. marinum* isolate NCIMB 1298 (n = 12) or NCIMB 1297 (n = 12). Four fish from each treatment were sampled at 0, 7, 11 and 14 days post-infection (d.p.i.) according to Table 3-1 to investigate the process of early granuloma formation over the first 14 d.p.i.

The second experiment was carried out to investigate granuloma formation over 6 month of infection. A total of 96 zebrafish were injected with either PBS (n = 48) or *M. marinum* NCIMB 1297 (n=48) and 8 fish per treatment were sampled at 28, 56, 84, 112, 140 and 168 d.p.i. according to Table 3-2.

After terminal anaesthesia as described in section Chapter 2.3.1., fish from both experiments were transferred into 10% neutral buffered formalin (NBF).

Table 3-1 Experimental set up for granuloma formation studies *in vivo*, including the total number of injected fish for each of the three treatments (PBS, *M. marinum* strain NCIMB 1298 and 1297) as well as the designated numbers of fish sampled at each of the 4 time points (d.p.i.).

Sampling days (d.p.i.)	PBS	*M. marinum* NCIMB 1298	*M. marinum* NCIMB 1297
0	4		
7	4	4	4
11	4	4	4
14	4	4	4
Total no.	**16**	**12**	**12**

Table 3-2: Experimental set up for granuloma formation studies *in vivo*, including the total number of injected fish for each of the two treatments (PBS, *M. marinum* strain NCIMB 1297) as well as the designated numbers of fish sampled at each of the 6 time points (d.p.i.).

Sampling days (d.p.i.)	PBS	*M. marinum* NCIMB 1297
28	8	8
56	8	8
84	8	8
112	8	8
140	8	8
168	8	8
Total no.	**48**	**48**

3.2.4 Sample preparation

Prior to processing, the majority of fish were transferred from 10% NBF into 95 % ethanol (ETOH) at 4°C for 4 h, followed by an 18 h overnight incubation in 0.5 M Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich Company Ltd, Dorset, UK), at 4°C to decalcify tissues according to the protocol of Aihara *et al.* (2008). An exception to the decalcification treatment was made for the fish collected after 14 d.p.i. Only 4 of the 8 fish sampled between 28 d.p.i. and 168 d.p.i. were decalcified, while the remaining 4 fish were kept in 10% NBF to enable suitable tissue samples for the von Kossa staining technique.

Sampled fish were processed and embedded in wax the next day following the protocol described in Chapter 2.4.1. Prior to sectioning the wax-embedded samples,

the paraffin blocks were trimmed in 20 µm sagittal sections until the first target organ (liver, spleen or kidney) was exposed. From that point 7 µm sections were made and subsequently transferred into a water bath and from there onto microscope slides. Sections were taken through the whole fish, focusing on having at least one of the target organs in a section. A minimum number of 20 sections was produced for each fish and an assortment of these slides was stained with a variety of different staining techniques.

3.2.5 Staining techniques

3.2.5.1 Haematoxylin and Eosin

Zebrafish sections were stained with haematoxylin and eosin stain (H&E) following the Institute of Aquaculture's in-house histopathology laboratory method, which was modified from Mayer's original mixture (Kiernan, 2008) by the addition of a washing step in Scottish tap water substitute and prolonged eosin staining step due to a variation in the eosin recipe. A minimum of 5 sections containing one or more of the target organs were stained.

Briefly, the sections were de-waxed in xylene for 5 min, transferred into 100% ethanol for 2 min, 100% methylated spirits for 1 min and washed under running tap water for 30 sec to 1 min. The sections were stained in Haematoxylin Z (Cellpath, Newtown, UK) for 5 min and subsequently washed under running tap water. Afterwards the sections were quickly dipped in 1% acid alcohol (1% hydrochloric acid in methylated spirits) 3-4 times, followed by three 1 min washing steps each with running tap water, Scotts tap water substitute (Cellpath) and an additional step with running tap water. After staining for 5 min with eosin (1 part Putt's Eosin with 8 parts 1% Eosin) (Cellpath), the sections were washed with running tap water for 30 sec to 1 min, with 100% methylated spirits for 1 min and with 100% alcohol for 3 min. A final clearing step for 5 min with xylene was carried out before mounting the slides with Pertex mounting media (Leica, Milton Keynes, UK). Nuclei appeared blue to

blue/black, cytoplasm and muscle fibres were stained deep pink, collagen was light pink and red blood cells as well as eosinophilic granules were bright orange or red.

3.2.5.2 Ziehl-Neelsen

The Ziehl-Neelsen staining technique is a bacteriological stain used to identify acid-fast organisms and works especially well for staining mycobacteria. A minimum of 3 sections, where possible sequential sections to those used in the H&E stain were stained. Briefly, the zebrafish sections were de-waxed for 5 min in xylene and afterwards transferred to 100% ethanol for 2 min, followed by a wash in 100% methylated spirits for 1 min. The sections were washed in running tap water for 30 sec to 1 min before being stained in filtered carbol fuchsin (VWR, Lutterworth, UK) for 60 min. After that, the slides were blotted on filter paper to removed excess liquid. The sections were subsequently rinsed briefly in 1% acid alcohol. The alcohol was repeatedly renewed until the sections appeared pale pink in colour. The sections were then washed under running water and immersed in 1% acidified methylene blue (1 part methylene blue (VWR), 1 part acetic acid, 20 parts ethanol, 80 parts distilled water (dH_2O) for 15 – 30 sec as a counterstain. Excess counterstain was removed by briefly washing the sections under running tap water. The sections were subsequently dehydrated and differentiated with a short wash in 100% ethanol. After a final bath for 5 min in xylene, the sections were mounted with Pertex. Acid fast bacilli were stained magenta.

3.2.5.3 Von Kossa stain

This technique was used to demonstrate abnormal deposits of calcium or calcium salt in tissue sections. Sections from non-decalcified fish and preferably sequential sections to the ones which were shown to be positive for granuloma by the H&E and Ziehl-Neelsen stain were stained using this technique.

Zebrafish sections were de-waxed for 5 min in xylene and afterwards transferred in 100% ethanol for 2 min, followed by a wash in 100% methylated spirits

for 1 min. The sections were washed in water for 30 sec to 1 min before washing several times in dH_2O. The area on the slide containing the zebrafish section was framed with a PAP pen to retain the stain on the section. Prior to incubation, 2% silver nitrate (aqueous) (SPI supplies, Loughborough, UK) was pipetted onto the section and the slides were exposed to bright light by placing them under a desk lamp for two hour. Completion of the reaction was confirmed macroscopically and microscopically, after which the sections were washed in several changes of dH_2O, followed by a 5 min treatment with 2.5% sodium thiosulphate (VWR). The sections were then washed thoroughly in tap water before applying the counterstain. Light neutral red (VWR) was chosen as the counterstain, which was applied for 20 sec before being washed off with tap water. Finally the sections were dehydrated in 100% ethanol, cleared in xylene and mounted with Pertex. Calcium deposits appeared brown to black in colour while the other tissues appeared red. Decalcified fish were used as negative controls and additional negative controls were prepared by treating non-decalcified fish with 10% formic acid (Sigma-Aldrich) for 10 min before incubating with silver nitrate.

3.2.5.4 Masson's Trichrome stain

Masson's trichrome stain uses three dyes including one that is specific for collagen. The principal use for trichrome is to differentiate collagen from other eosinophilic structures, such as muscle fibres. Similar to the section chosen for the von Kossa, only sequential sections to the ones tested positive for granuloma were utilised for this staining technique.

The slides with the zebrafish sections were de-waxed for 5 min in xylene and transferred to 100% ethanol for 2 min, followed by a wash in 100% methylated spirits for 1 min. The sections were washed in tap water before staining with the nuclear stain celestine blue (0.5 g celestine blue B (VWR), 5 g ferric ammonium sulphate (Sigma-Aldrich), 14 ml glycerol (Sigma-Aldrich), 100 ml dH_2O) for 5 min. After incubation, the sections were washed in tap water before staining them with Haematoxylin Z for 5 min.

They were subsequently washed for 5 min under running tap water. The nuclear stain was then differentiated by dipping the section 3-4 times into 1% acid alcohol and washing them for 5 min in running tap water, before transferring them into a Coplin jar with distilled water (dH_2O). The sections were stained with cytoplasmic stain (2 parts of 1% ponceau de xylidine (Sigma-Aldrich) in 1% acetic acid and 1 part 1% acid fuchsin (VWR) in 1% acetic acid) for one hour. Prior to use the cytoplasmic stain was diluted 1:10 with 1% acetic acid. After incubation, the sections were rinsed in dH_2O and then differentiated in 1% phosphomolybdic acid (Sigma-Aldrich) for 1-2 min. After another rinse in dH_2O, the sections were counterstained with 2% light green (VWR) in 1% acetic acid for 2 min and washed well in 1% acetic acid for 1-2 min. Finally, the sections were blotted to remove excess liquid before dehydrating in 100% ethanol, clearing in xylene and mounting with Pertex.

Nuclei were stained black, while muscles, red blood cells, fibrin and some cytoplasmic granules stained red, and collagen, reticulin and amyloid and mucin stained green.

3.2.6 Microscopic analysis of zebrafish sections

Microscopic analysis was performed blind by covering the slide identities prior to examination. The slides were examined under an Olympus light microscope BX51 and using AxioVision image capture software Version 4.0 (AxioVs40 V 4.8.2.0) (ZEISS, Jena, Germany). The extent of disease progression and granuloma formation was determined by assessing the total number of granulomas and affected organs in the Ziehl-Neelsen stained sections. For the total number of granulomas, no distinction was made with respect to the various granuloma types. Analysis of granuloma formation was adapted from Parikka *et al.* (2012).

In addition, each time point was subsequently analysed more closely including detailed description of types of granuloma and affected organs. The various types of granuloma were distinguished into four major types of granuloma:

(1) non-necrotic: early association of macrophages, which accumulate to a solid or non-necrotic granuloma

(2) necrotic: a sharply circumscribed nodule that mainly consists of modified macrophages with beginning of necrosis in the centre recognizable by nuclear break-down

(3) caseating necrotic: necrotic granuloma with progressing cellular break-down in the centre. This necrotic centre softens without any cellular outline and is referred to as caseum or caseating necrosis due to its cheese-like appearance resulting from extensive cell death

(4) multi-centric granuloma: necrotic or caseating necrotic granuloma with two or more necrotic centres, circumscribed by fibroblasts

Furthermore, early signs of calcification and fibrosis might be detectable in necrotic, caseating necrotic and multi-centric granulomas.

3.2.7 Statistical analysis

Statistical analysis was carried out using GraphPad Prism v.5 (GraphPad software). Significant differences in granuloma numbers and affected organs over time were determined by the non-parametric Kruskal-Wallis test. The log-rank Mantel-Cox test was applied to determine significant difference in the mortality curves of PBS and *M. marinum* NCIMB 1297 injected fish.

3.3 *Results*

3.3.1 Host death and survival of injected zebrafish

No differences were observed between the zebrafish challenged with *M. marinum* isolate NCIMB 1298 and the control, mock-infected fish. The survival of fish injected with *M. marinum* isolate NCIMB 1297 and the control fish is illustrated in Fig. 3-2 and is referring to unexpected mortalities during the experimental set up. Mortalities were first recorded in zebrafish injected with *M. marinum* isolate NCIMB 1297 from 60 d.p.i. and by three months post-infection, 18 of these fish had died or were culled due to severity of symptoms.

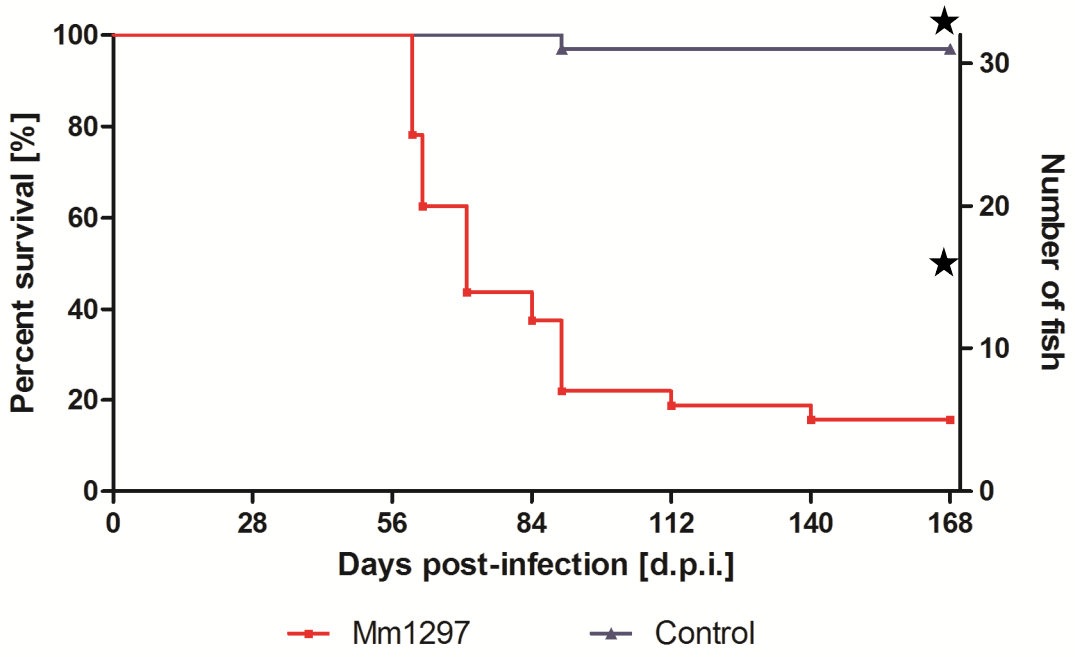

Figure 3-2: Percent survival of challenged and control zebrafish over experimental period. The graph shows the survival rate in percent of *M. marinum* NCIMB 1297 injected (red line) and mock injected control fish (blue line), only considering unexpected death over an experimental timeline of 168 days. Fish removed at sampling points are not included. Investigation of NCIMB 1297 and PBS injected fish were investigated over the full experimental timeline of 168 days. Significant difference in the survival curves is marked by a star ($p<0.001$).

Based on the limited number of fish remaining, the decision was made to postpone all following sampling points to guarantee a minimum number of fish for the six month post-injection sampling point which was crucial for re-infection challenge and microarray analysis (Chapter 5).

By the end of the challenge period of 6 months, 27 out of 32 of the NCIMB 1297 infected fish had died due to mycobacteriosis (84.4%). The sick fish displayed signs of listlessness and spent most of the time on the bottom of the tank or showed signs of disoriented swimming. Badly affected fish revealed a heavily swollen abdomen with prominent blood vessels suggesting inflammation. In comparison, only 1 of the uninfected control fish had died (3.1%).

3.3.2 Granuloma pathology, progression and bacterial distribution

3.3.2.1 Control (mock injected) fish

All fish examined from the control group that had been injected with PBS, showed no signs of granuloma formation over the entire experimental period. No mycobacteria were evident with Ziehl-Neelsen staining at any of the time points examined.

3.3.2.2 Fish injected with *M marinum* strain NCIMB 1298

Similar to the mock-injected fish, the fish challenged with *M. marinum* isolate NCIMB 1298 showed no visible signs of granuloma formation when examined at 7 and 11 d.p.i. At 14 d.p.i., one out of the 4 fish examined possessed a granuloma.

3.3.2.3 Fish injected with *M marinum* strain NCIMB 1297

The numbers of granulomas, as well as the affected organs, were determined to gain an overview of the progression of the disease over time (Table 3-1, Fig. 3-3 and 3-4).

The total numbers of granulomas per fish increased over time (Table 3-1, Fig. 3 3). The total number of granulomas at 11, 14 and 28 d.p.i. were significantly lower

(0.17 ± 0.11, 1.0 ± 0.53 and 5.25 ± 1.53, respectively) than at 56 d.p.i. (60.42 ± 11.69, $p < 0.0001$) but no significant difference could be found between 11, 14, and 28 d.p.i., although an increasing trend in the number of granulomas was observed.

Regarding the number of organs affected (Fig. 3-4), an increasing trend was also detectable. With regard to the significant differences between the four time points, the results revealed a significantly higher number of affected organs at 56 d.p.i. (4.67 ± 0.38, $p < 0.0001$) compared to 11 and 14 d.p.i. (0.17 ± 0.11 and 0.78 ± 0.32) but not to 28 d.p.i. (2.25 ± 0.55 respectively). No significant differences were found between 11 and 14 in the number of organs affected.

Table 3-1: Histological analysis of granuloma formation in adult zebrafish experimentally infected with *M. marinum* NCIMB 1297 at 11, 14, 28 and 56 days post-infection (d.p.i.).

	Time point (No.of examined slides)																							
	11 d.p.i.												14 d.p.i.											
Organs examined	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
Liver	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-			
Peritoneum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Head kidney	-	-	-	-	-	-	-	-	-	1	-	-	-	3	-	-	-	-	-	-	-			
Kidney	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Pancreatic tissue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Connective tissue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Adipose tissue	-	-	-	-	-	-	-	-	-	-	-	-	1	1	1	1	1	-	-	-	-			
Gonads (Ovaries / Testes)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Brain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Head	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Eye	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Total granuloma number	1	0	0	0	0	0	0	0	0	1	0	0	1	5	1	1	1	0	0	0	0			
	28 d.p.i.												56 d.p.i.											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7*	8*	9*	10*	11*	12*
Liver	1	2	7	6	4	-	-	-	-	1	-	-	-	1	4	12	15	7	13	15	25	2	5	4
Peritoneum	2	-	-	-	-	-	-	-	-	-	1	-	4	-	-	5	3	-	-	2	1	-	-	
Head kidney	4	2	1	1	2	-	-	-	-	-	-	-	-	1	3	8	9	15	17	16	-	105	87	70
Kidney	5	3	1	-	-	-	1	-	-	1	1	1	5	2	-	24	19	11	19	14	20	19	30	22
Pancreatic tissue	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Connective tissue	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adipose tissue	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	2	-	-	-	-	3
Gonads (Ovaries / Testes)	6	-	-	-	-	-	-	-	-	-	-	-	5	3	3	-	-	-	-	-	-	-	-	-
Brain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-	-	-	-	-	-
Head	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	5	3	-	2	2	3	-	-
Spleen	-	-	-	-	-	-	-	-	-	1	2	1	-	-	3	4	8	-	2	5	13	1	-	-
Eye	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	4	4	5	-	-	
Total granuloma number	18	7	9	7	6	0	1	0	0	9	4	2	14	7	13	64	59	36	57	58	66	130	122	99

Granulomas were counted in a total number of 12 sagittal sections from whole zebrafish, derived from 4 different fish per time point and stained with Ziehl-Neelsen. In case of 14 d.p.i. only 3 fish were available. Total number of granulomas per section was determined in addition to number of granulomas in affected tissue and organs.

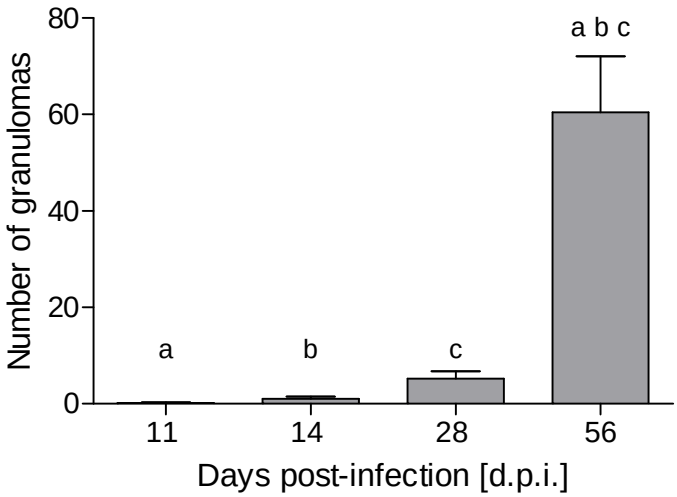

Figure 3-3: Average number of granulomas observed in zebrafish sections over time following injection of adult fish with *M. marinum* NCIMB 1297. The average number ± standard error of granulomas counted in the tissue sections at 11, 14, 28 and 56 days post-infection (d.p.i.). Four individuals per time point of *M. marinum* NCIMB 1297 injected fish stained with Ziehl-Neelsen (except 14 d.p.i., n=3). In total, 3 sections per fish were examined per time point. Statistical significant difference ($p<0.05$) between time points were determined by non-parametric Kruskal-Wallis test and visualised by being labelled with the same letter

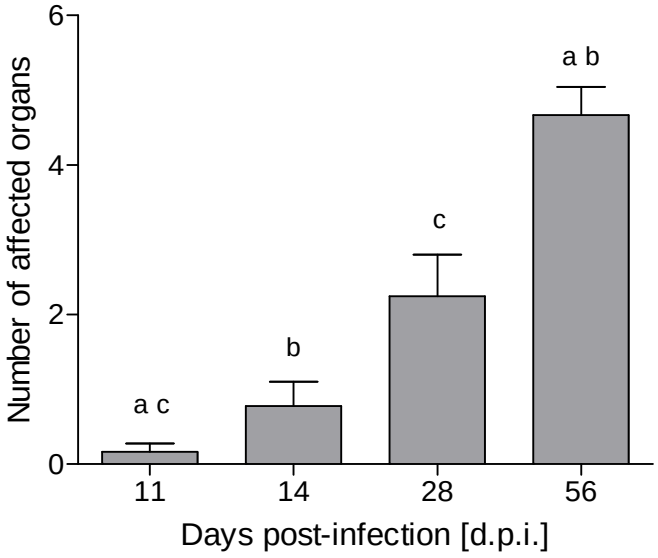

Figure 3-4: Average number of affected organs in zebrafish sections over time following injection of adult fish with *M. marinum* NCIMB 1297. The average number ± standard error of affected organs found in the examined zebrafish sections at 11, 14, 28 and 56 days post-infection (d.p.i.). Four individuals per time point of *M. marinum* NCIMB 1297 injected fish stained with Ziehl-Neelsen (except 14 d.p.i., n=3). In total, 3 sections per fish were examined per time point. Statistical significant difference ($p<0.05$) between time points were determined by non-parametric Kruskal-Wallis test and visualised by being labelled with the same letter.

At 11 d.p.i., two out of the four fish examined displayed one granuloma each, located in the head kidney (Fig. 3-5.a+b) or in the ovaries of the examined fish. The granuloma at this stage was non-necrotic containing only few mycobacteria (black arrow), which were engulfed by cells forming the granuloma (visualized as purple rods, white arrows).

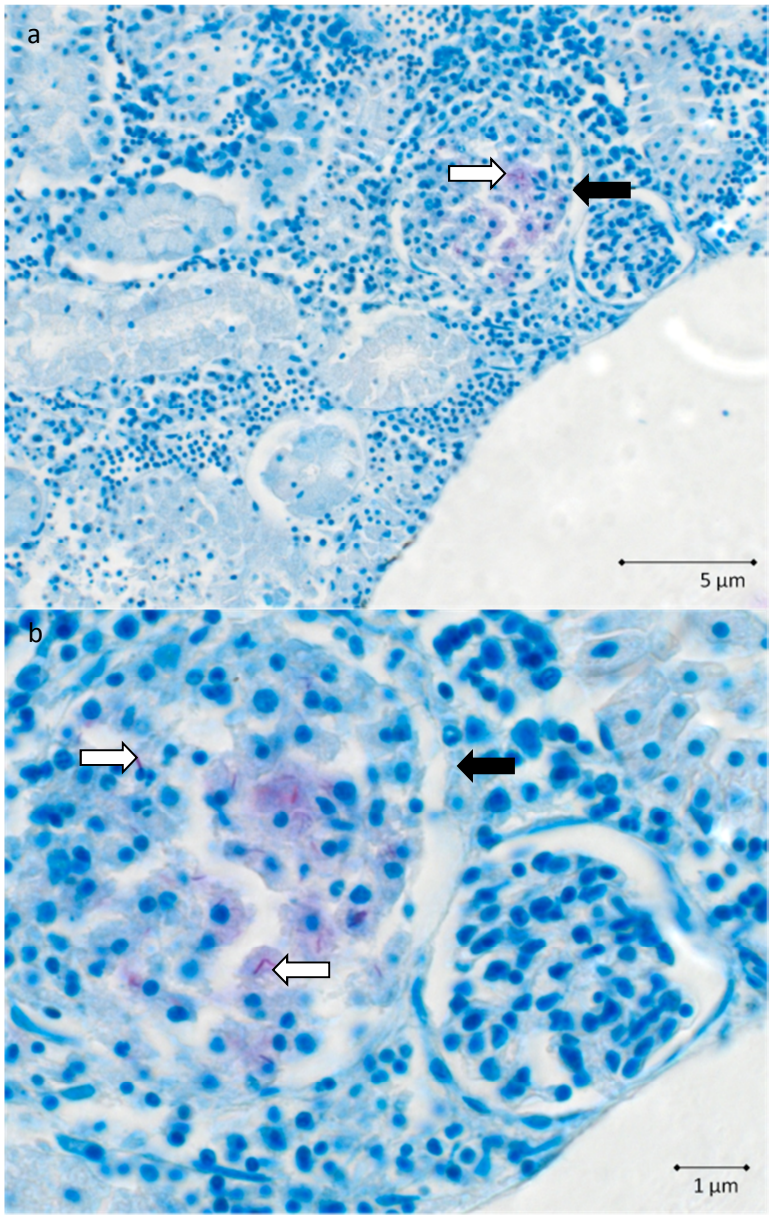

Figure 3-5: Light micrograph of Ziehl-Neelsen stained section of a non-necrotic granuloma observed in the kidney of an adult zebrafish experimentally infected with *M. marinum* NCIMB 1297 at 11 d.p.i. under 40x (a) and 100x (b) magnification. Black arrow indicate granuloma in a, white arrows indicate positively stained mycobacteria in b.

At 14 d.p.i., granulomas were found in two out of the three fish examined. Most of the sections analysed at this time point revealed only one granuloma, which was located in the adipose tissue, with the exception of one section in which a total of five granulomas was observed in kidney, liver and the adipose tissue. The granulomas were well-organized, non-necrotic granulomas (black arrows) with mycobacteria contained inside the granuloma visualized as purple rods (white arrows). Early signs of necrosis were detectable within these granulomas, where destroyed cells were still recognisable between the debris (black dashed arrows) (Fig. 3-6. a-d). No bacteria could be detected outside of granulomas.

At 28 d.p.i., all fish examined displayed granulomas. The number of granulomas per section increased in these fish relative to 14 d.p.i., ranging from 1 to 18. The number of organs affected also increased over time including kidney, liver, spleen, gonads, pancreatic tissue, connectice tissue, peritoneum and adipose tissue (Fig. 3-7 a-h). The granulomas were well-organized, non-necrotising and necrotising. The necrotising granulomas showed partial necrosis in the centre. The mycobacteria were contained inside the granulomas and a cuff of elongated cells, possibly fibroblasts, surrounded the granuloma and separated it from the connecting tissue. The number of bacteria was quite low in both types of granuloma and it was possible to distinguish individual rods.

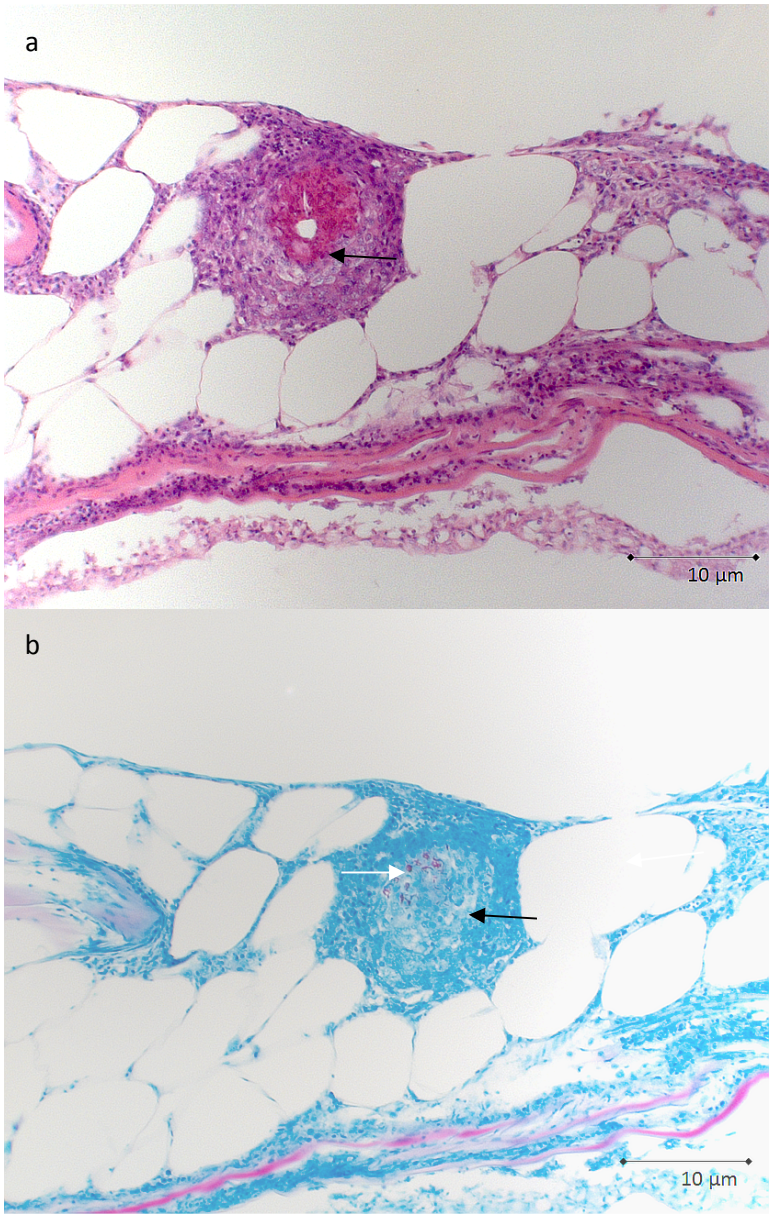

Figure 3-6: Light micrograph of granulomas observed in the peritoneum of zebrafish experimentally infected with *M. marinum* NCIMB 1297 at 14 d.p.i. Necrotic granuloma (black arrow) in infected zebrafish visualised with H & E (a) and Ziehl-Neelsen (b) stain under 10x magnification. Bacteria are visualised as purple rods (white arrow) in the Ziehl-Neelsen stained sections.

At 56 d.p.i., granulomas were found in all examined sections. The number of granulomas per section varied from 7 to 130. The sections with a low granuloma count revealed high numbers of free bacteria, which complicated the counting process due to difficulties in differentiating granulomas from bacteria-affected tissue. Similar difficulties were encountered in the determination of organs involved. Overall, the organs affected at this stage of the disease included kidney, liver, spleen eye, head, adipose tissue, gonads, brain and peritoneum. Besides the obvious increase in the granuloma formation, the bacterial load also increased, not only within the granulomas (Fig. 3-8 a-f), but also as freely disseminated bacteria in the areas surrounding the granuloma. The granulomas were predominately necrotising, with high bacterial load inside a single or multiple centres, but non-necrotising granulomas were also present (Fig. 3-9 a-e). An example of the variety of granulomas found in the liver of the same fish is illustrated in Fig. 3-9b showing non-necrotising, necrotic and multi-centric granulomas in close proximity.

Overall, few lymphocytes were detectable in the granulomas at any stages of the infection.

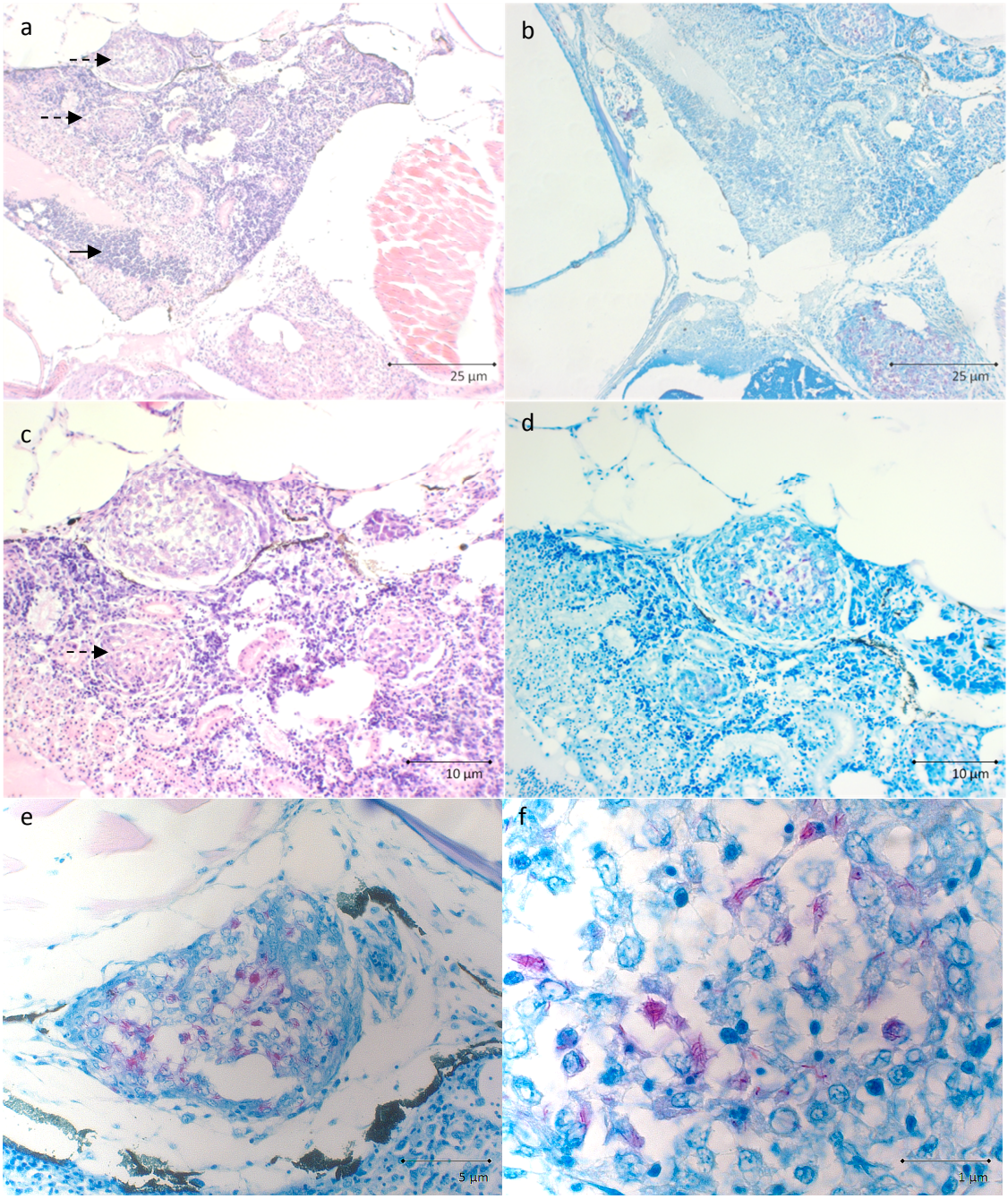

Figure 3-7: Granulomas observed in the (a-d) head kidney at 28 d.p.i. of adult zebrafish experimentally infected with *M. marinum* NCIMB 1297. Progression of disease showed necrotic non-necrotic (arrow) and partial necrotic granuloma (dashed arrow) in the kidney of infected fish visualised with (a, c) H & E and (b, d, e, f) Ziehl-Neelsen stain under (a, b,) 10x, (c, d) 20x, (e) 40x and (f) 100x magnification.

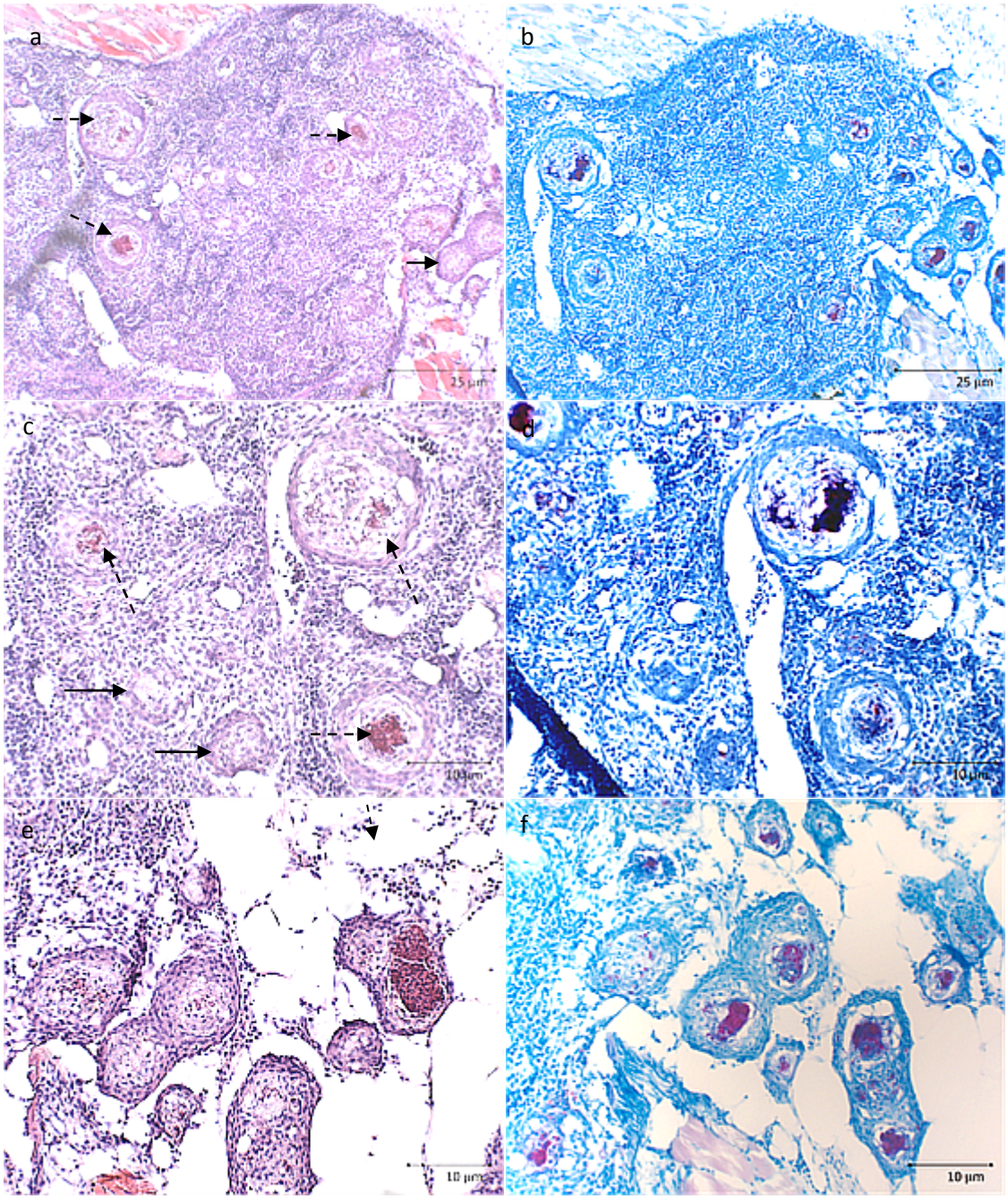

Figure 3-8: Granulomas observed in the (a-d) head and (e-h) anterior kidney at 56 d.p.i. of adult zebrafish experimentally infected with *M. marinum* NCIMB 1297. Progression of disease showed necrotic (dashed arrow) and non-necrotic granuloma (arrow) in the head kidney of infected zebrafish, visualised with (a, c, e) H & E and (b, d, f) Ziehl-Neelsen stain under (a+b) 10x and (c-f) 20x magnification.

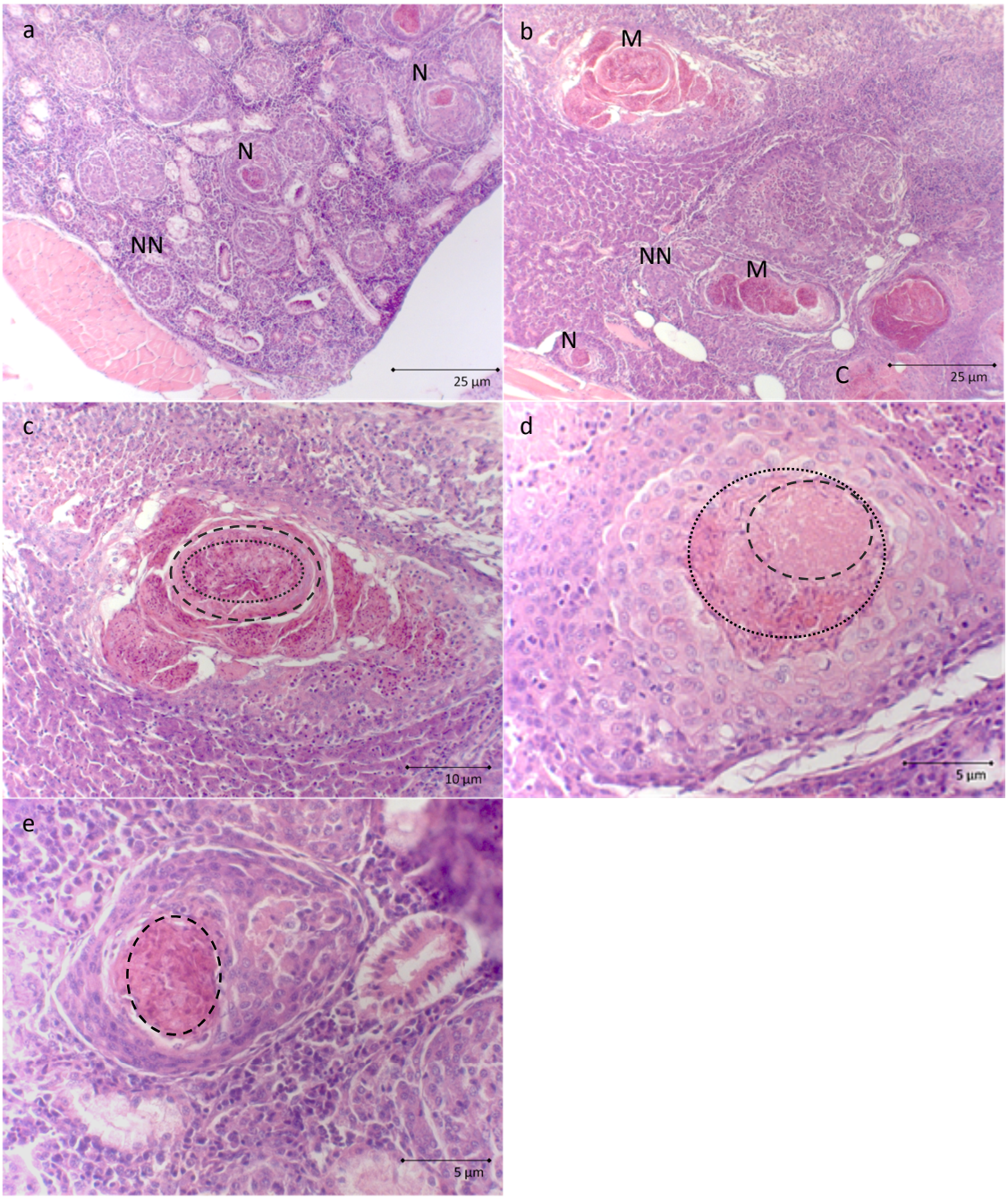

Figure 3-9: Different granuloma types observed at 56 d.p.i. in the head kidney of adult zebrafish experimentally infected with *M. marinum* NCIMB 1297. Illustrated are (a+b; NN) non-necrotic, (a, b, e; N) necrotic, (d; C) caseous and (b+c; M) multicentric, granuloma types found in (a) kidney and (b-e) liver of infected zebrafish visualised with H & E stain under (a+b) 10x, (c) 20x and (d+e) 40x magnification. Dashed lines indicate areas with caseum while dotted lines indicate necrosis (c-e)

3.3.3 Evidence of calcification and/or fibrosis in the granuloma

Sections tested positive for the presence of mycobacteria and granulomas were further tested for presence of calcification and/or fibrosis.

No evidence of calcification was detectable in any of the granulomas examined. The von Kossa stain applied to sections from non-decalcified fish i.e. in fish which had been infected for two months, showed no differences between control and infected fish. As additional negative controls, sections of infected fish were treated with formic acid to remove calcification prior to staining and decalcified infected fish were stained with von Kossa. None of the sections displayed any evidence of calcification within the granuloma (Fig. 3-10 a-d).

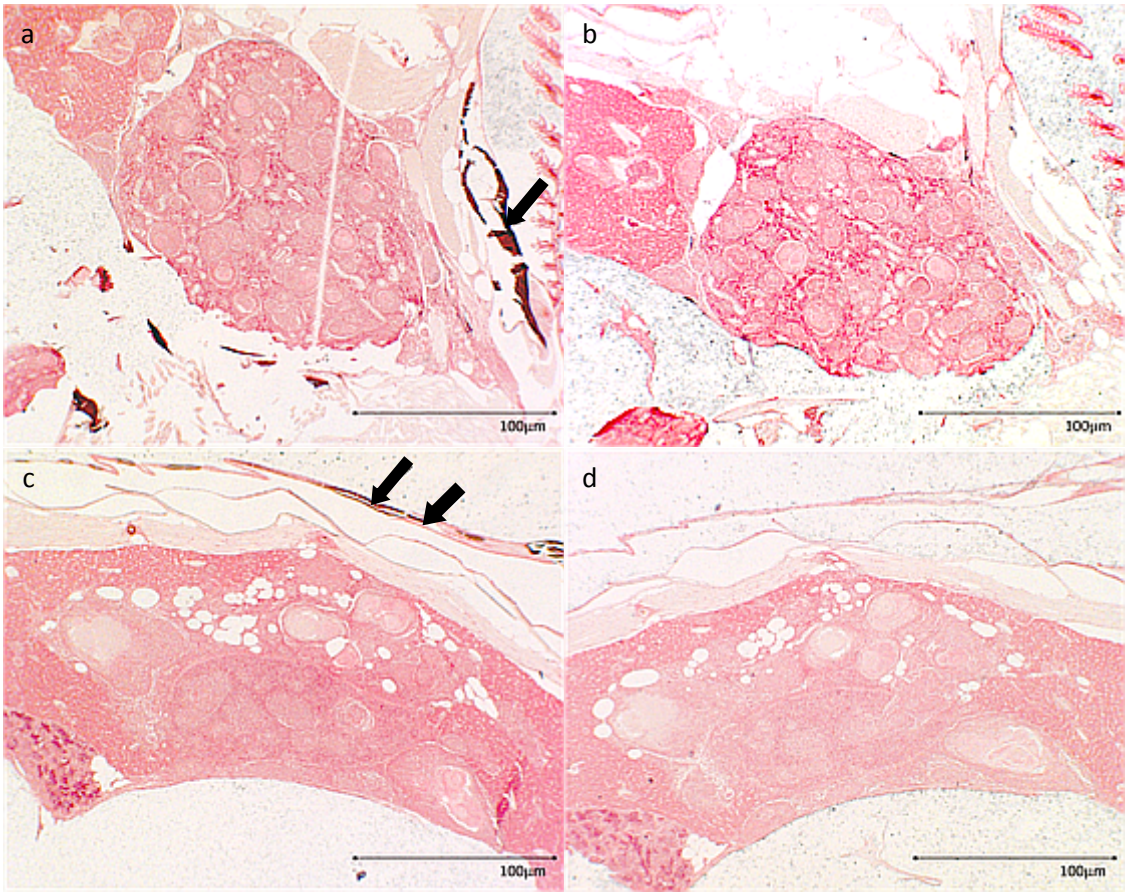

Figure 3-10: Granulomas observed at 56 d.p.i. and stained with von Kossa stain for the presence of calcium deposits in adult zebrafish experimentally infected with *M. marinum* NCIMB 1297. Illustrated are (a+b) head kidney and (c+d) liver of infected zebrafish (a+c) untreated or (b+d) pre-treated with formic acid under 4x magnification. Arrows indicate sites of positive reaction of the von Kossa stain at the zebrafish spine (a+c)

The existence of fibrosis was determined by applying the Masson's trichrome stain to sections containing granulomas. At 56 d.p.i., granulomas displayed early signs of fibrosis, most distinctively in the liver and kidney (Fig. 3-11 a-f). Granulomas were surrounded by thickening areas of fibrotic connective tissue (visualized by green stain) with progression from outer granuloma areas into the centre of the granuloma (Fig. 3-11 d).

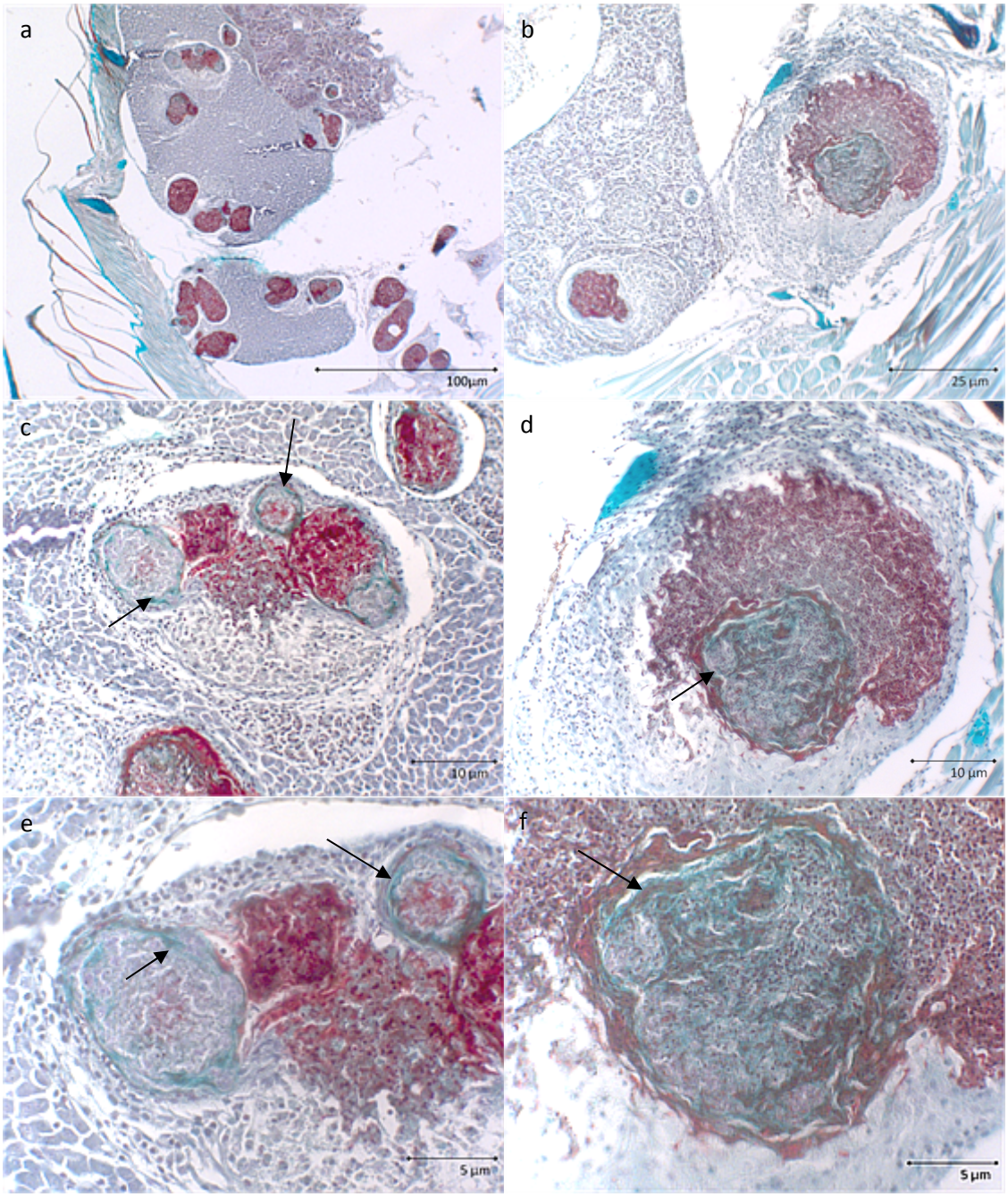

Figure 3-11: Granuloma observed at 56 d.p.i. with Masson's trichrome stain for the presence of collagen/ fibrotic connective tissue in adult zebrafish experimentally infected with *M. marinum* NCIMB 1297. Illustrated are granuloma formations found in the (a, c, e) liver and (b, d, f) kidney of infected fish under (a) 4x, (b) 10x, (c+d) 20x and (e+f) 40x magnification. Arrows indicate sites of progressive fibrosis.

3.4 *Discussion*

3.4.1 Difficulties during histological examination

The small size of the zebrafish meant it was possible to examine granuloma formation, disease progression and dissemination of bacteria within the same individual fish. Furthermore, the ability to produce sagittal serial sections from the same fish, allowed the examination of the same granuloma on more than one slide, giving the opportunity of applying different staining techniques to examine the granuloma for different features such as pathogen type, e.g. *Mycobacterium spp* or *Francisella spp*., or the presence of calcification and/ or fibrotic connective tissue. Despite these advantages, the creation of a continuous ribbon of serial sections from a whole fish also presented some challenges.

For example, the bone structure in the head and spine of the fish damaged the blade, which in turn broke the tissue as it cut sections (Callis 2008). Decalcification of the fish prior to processing helped to overcome this damage. The decalcification step allowed better sections to be made and maximized the life of the blade, but it prohibited the examination of calcification in the granuloma. Another side-effect that arose from the fixation of the whole fish was that the side of the swim bladder was a hollow space within the fish, making it difficult to transfer the section to the water bath. Depending on the size of this hollow, the sections tended to disintegrate during sectioning especially if the blade was damaged, when transferring them into the water bath or due to water movement in the water bath.

3.4.2 Effect of injected *M. marinum* strain on disease outcome

Over the course of the trial, fish injected with *M. marinum* NCIMB 1297 (ATCC 927) developed granulomas and this organism was therefore considered to be virulent in the zebrafish. In contrast, isolate NCIMB 1298, the other *M. marinum* isolate used in this study, caused no visible response, macroscopically and microscopically within the first 14 d.p.i., with the exception of a single granuloma in one fish. This one granuloma might be a false positive. At the beginning of the sectioning of the zebrafish, the water in the water bath was not changed when changing from one challenge group to another and might have caused cross-contamination on the slides due to remains of infected tissue in the water.

The only source referring to the infectious nature of NCIMB 1298 is the NCIMB homepage which categorises the isolate as only virulent to neon tetra (*Paracheirodon innesi*) (NCIMB Ltd 2013). Based on this statement and in agreement with the finding of this study, NCIMB 1298 appeared to be non- virulent in the zebrafish. Other sources only refer to this strain for its production of extracellular products (Chen *et al.* 1997; 1998). Differences in the infectious nature of different *M. marinum* isolates have been previously reported and this seems to have a significant influence on disease progression in zebrafish (Talaat *et al.* 1998; Prouty *et al.* 2003; van der Sar *et al.* 2004a; Swaim *et al.* 2006; Watral and Kent 2007; van der Sar *et al.* 2009; Parikka *et al.* 2012).

Depending on the isolate used, *M. marinum* can cause an acute or chronic disease, and isolates causing an acute disease appear to belong mainly to a cluster isolated from man, whereas chronic disease is predominately produced by *M. marinum* isolated from poikilotherms (van der Sar *et al.* 2004a; 2009). In contrast to the findings of van der Sar *et al.*, Swaim and her group (2006) managed to infect adult zebrafish with a human-derived isolate resulting in a chronic disease progression. A recent publication on the infectious nature of *M. marinum* isolate ATCC 927 (NCIMB 1297)

injected into adult zebrafish revealed once again that the dose applied to the zebrafish has a significant influence on the progression of the disease (Parikka *et al.* 2012). A dose as low as 34 cfu per fish was enough to cause a long-term, stable latent infection, while a higher dose of the same isolate was likely to induce a chronic progressive disease. The results of Parikka *et al*. (2012) agree and support the findings of Swaim *et al.* (2006), that human-derived strains are more virulent than strains originating from fish, which require a higher infection dose to achieve a similar disease progression in zebrafish.

The infectious dose used in the current study was approximately 250 cfu per fish, which led to a chronic progressive disease which differed to that described by Parikka *et al.* (2012) and perhaps fell between the two experimentally produced disease stages described in their study. Dose-dependent changes in the outcome of mycobacterial infections have been explored widely using zebrafish, goldfish and medaka as model organisms (Talaat *et al.* 1998; Prouty *et al.* 2003; van der Sar *et al.* 2004a; Swaim *et al.* 2006; Broussard and Ennis 2007; Watral and Kent 2007; van der Sar *et al.* 2009; Parikka *et al.* 2012). Regarding the clustering of *M. marinum* strains into those which cause an acute infection from human-derived isolates, or a chronic disease resulting by isolates derived from poikilothermic species, this statement is true if the same dose is injected into zebrafish. By adjusting the infective dose with respect to the isolate origin, it is possible to induce chronic and possibly latent mycobacterial infection with any isolate, but further investigations have to be carried out to validate this statement.

3.4.3 Mortality of *M. marinum* NCIMB 1297 infected fish

Zebrafish challenged with *M. marinum* isolate NCIMB 1297 (ATCC 927) displayed a high end point mortality of 84.4 %. No mortalities were observed over the first two months post-infection, and then there was rapid onset of mortality. By 3 m.p.i., nearly 70% of the fish were dead. With an infective dose of 2.5 x 10^2 cfu/ fish, end

point mortality between 25% and 63% would be expected according to the findings from Parikka *et al.* (2012), who found this level of mortality in zebrafish infected with 3.4 x 10^1 cfu and 2.03 x 10^3 cfu of the same isolate, respectively. Similar results have been published by Prouty *et al*. (2003) who described an end point mortality after 18 weeks of ~ 60% for fish infected with 1.0 x 10^3 cfu/ fish of *M. marinum* isolate ATCC 927 and no mortality in fish injected with 1.0 x 10^3 cfu.

Personal observations suggest that most of the dying fish were females at the peak of sexual maturation and therefore full of eggs. Sexual maturation, especially the late stages of maturation are believed to be immunosuppressive in fish. Studies on oestrogens, androgen hormones like testosterone, and cortisol in fish have shown the immunosuppressive nature of these hormones (Wang and Belosevic 1995; Harris and Bird 2000; Schreck *et al.* 2001; Schreck 2010; Casanova-Nakayama *et al.* 2011). There is a high possibility that this suppression led to a natural re-infection of the female zebrafish with *M. marinum* in the current study, this agreeing with similar findings in maturing female goldfish and rainbow trout, which showed an enhanced susceptibility to pathogens (Wang and Belosevic 1995; Casanova-Nakayama *et al.* 2011). Removing dead or dying fish from the tank can also be stressful, leading to further immunosuppression, evaluated through levels of the stress hormone cortisol. Ramsay *et al*. showed that netting and handling stress, even for a short time period, increased the blood cortisol levels in zebrafish (Ramsay *et al.* 2006; 2009a). Removing dead fish also revealed that most of the dead fish were victims of post-mortem cannibalism, which is not uncommon in this fish species (Smith and Reay 1991; Astrofsky *et al.* 2000; Harriff *et al.* 2007). Ingesting dead infected fish tissues may have also led to reinfection (Jacobs *et al.* 2009). Furthermore, bacteria would have been released from dead fish not yet removed from the tank, facilitating the uptake of bacteria through the gills and mouth of the remaining fish. This, together with late-stage maturation, the netting stress and the cannibalism might have triggered a fatal circle of re-infection, dying and re-infection. This may explain the mortalities recorded

during this study, as other research groups showed that low infection dose did not trigger such a high end-point mortality (Prouty *et al.* 2003; Parikka *et al.* 2012).

3.4.4 Disease progression and granuloma formation

The zebrafish in the current study, injected with 2.5×10^2 cfu, developed few granulomas, and few organs were affected during the first two weeks of infection. This was followed by a significant increase in both granuloma numbers and organs affected, after one month. The zebrafish developed a chronic progressive disease which apparently started in the peritoneum with most granulomas detected in this tissue and may reflect the fact that the initial injection of the fish was into the intraperitoneal cavity. The classical model of granuloma formation took place in which localised resident phagocytes were the first immune cells to react to the pathogen, and subsequently engulf the bacteria. The individual infected macrophages were then surrounded by aggregates of mononuclear phagocytes to form a non-necrotic granuloma (Cosma *et al.* 2003; Basaraba 2008; Barry *et al.* 2009; Ramakrishnan 2012; Reece and Kaufmann 2012). At this stage, the zebrafish appear to have developed an equivalent to a primary lesion when compared to mammalian granuloma models (Basaraba 2008; Ramakrishnan 2012).

Recently it has been suggested that the granulomas may not 'wall off' the bacteria from the surrounding tissue (Ramakrishnan 2012), as has been believed for many decades. Several studies confirmed that mycobacteria exploit granuloma formation and turn the immune response against the host, using both granuloma and host response for their proliferation and dissemination (Davis *et al.* 2002; Volkman *et al.* 2004; Davis and Ramakrishnan 2009; Volkman *et al.* 2010; Ramakrishnan 2012). It appears that the granuloma formation and its structure is a highly dynamic environment with a constant exchange and transmission of immune cells between the central area and surrounding layers (Cosma *et al.* 2003; Davis and Ramakrishnan 2009; Ramakrishnan 2012). This dynamic structure to the granuloma aids the

dissemination of the bacteria. Newly arriving macrophages can move through the granuloma and phagocytose the infected and dead macrophages, by which the new macrophages also become infected (Ramakrishnan 2012). It has been shown that mycobacteria are able to modulate this phagocytic behaviour to their advantage by attracting newly arriving macrophages to move close to the infected ones (Davis and Ramakrishnan 2009; Ramakrishnan 2012). Furthermore, it has been shown that some of the infected macrophages were able to leave the primary granuloma, which establish secondary granulomas and thereby help the dissemination of the disease (Clay *et al.* 2007; Davis and Ramakrishnan 2009; Ramakrishnan 2012).

The results of this study showed that after one month, the disease had spread to kidney, liver, ovaries and other organs with non-necrotic and partial necrotic granulomas appearing, and after two months even more organs were affected. The granulomas detectable after two months were mostly necrotic, although some non-necrotic ones were also observed. Furthermore, the bacterial load was increased significantly in the necrotic lesions and caseation became detectable. In addition, multicentric granuloma appeared and thin fibrotic cuffs surrounded the necrotic granulomas. Necrosis is believed to be a feature of more mature granulomas with regard to human tuberculosis infections (Basaraba 2008), and necrotic granulomas develop from non-necrotic ones with the necrotic region consisting of dead infected cells (Ramakrishnan 2012). In humans and some mammalian tuberculosis models, the necrotic progress starts after 3-4 weeks post-infection (Basaraba 2008), which agrees with the finding here in which the first noticeable partial necrosis was detectable after 4 weeks. These results were further supported by the findings of Swaim *et al.* (2006), who observed necrosis beginning after 8 weeks post-infection in their zebrafish model, subsequently followed by multicentric necrotic lesions and caseating necrotic lesions after 16 weeks of infection. The development of necrosis and caseating necrosis is an important feature in granuloma formation but is still poorly understood (Basaraba 2008; Tobin and Ramakrishnan 2008; Ramakrishnan 2012). It has been shown that not all

tuberculosis models or natural *Mycobacterium* hosts display this feature. In mammalian models infected with *M. tuberculosis*, rabbits, guinea pigs and macaques develop caseating granulomas during infection, while the majority of mice showed poorly organised macrophage and lymphocyte aggregates without any sign of caseation (Flynn 2006; Tobin and Ramakrishnan 2008; Ramakrishnan 2012). In humans, both strains *M. tuberculosis* and *M. marinum* lead to the development of necrotic lesions (Travis *et al.* 1985; Tobin and Ramakrishnan 2008). In ectotherms which have been injected with *M. marinum*, necrosis has been detected in goldfish, zebrafish and medaka as well as other fish species and toads (Huminer *et al.* 1986; Asfari 1988; Talaat *et al.* 1998; Swaim *et al.* 2006; Broussard and Ennis 2007; Gauthier and Rhodes 2009; Jacobs *et al.* 2009), but not in leopard frog which developed a long-term subclinical disease with non-necrotic lesions even when injected with high infection doses (Ramakrishnan *et al.* 1997; Bouley *et al.* 2001). This host specificity for the formation of necrotic granulomas suggests that the necrosis development may involve certain host factors (Cosma *et al.* 2003; Swaim *et al.* 2006; Basaraba 2008). Evidence that necrosis in granulomas is not only a host induced mechanism and is also partially induced by the *Mycobacterium* spp. has been provided by Swaim *et al.* (2006) and Gao *et al.* (2004) who showed that *M. marinum* in zebrafish and *M. tuberculosis*-infected mice prolonged the survival of their hosts when the RD1 locus in the *Mycobacterium* genome was absent, which encodes the virulence of the strains. In addition, the zebrafish in that experiment displayed a higher number of non-necrotic granulomas compared to the fish infected with the wild-type *M. marinum* strain. Similar observations have been found in mice infected with BCG or *M. tuberculosis* (Junqueira-Kipnis *et al.* 2006; Kurenuma *et al.* 2009). Injection with BCG, which is lacking the RD1 locus resulted in the absence of necrosis while injection with *M. tuberculosis* caused necrosis in the lungs of the infected mice (Junqueira-Kipnis *et al.* 2006). A connection between the appearance of necrosis and increasing bacteria number in the granuloma has also been reported (Swaim *et al.*

2006; Basaraba 2008; Parikka *et al.* 2012; Ramakrishnan 2012). Necrosis seems to assist bacterial dissemination and the spread of the disease. In the necrotic cell death event, the dying infected macrophages releases ingested bacteria into the extracellular environment, permitting and supported bacterial growth and disease dissemination (Ramakrishnan 2012). Higher bacteria numbers were also detected in the necrotic granuloma centres in this study, supporting the idea that there is a relationship between necrosis and mycobacterial growth.

The granulomas found in the present study contained few lymphocytes, similar to those described in the study of Swaim *et al.* (2006). A low lymphocyte number is generally observed in fish and frogs infected with *M. marinum* (Cosma *et al.* 2003), as seen in goldfish (Majeed *et al.* 1981; Talaat *et al.* 1998) and leopard frogs (Bouley *et al.* 2001). Mammals on the other hand recruit large numbers of lymphocytes to maintain the structure of granuloma and restrict the infection caused by *M. tuberculosis* (Cosma *et al.* 2003), a feature which can be also detected in human granuloma caused by *M. marinum* (Travis *et al.* 1985). Swaim *et al.* (2006) suggested that the reason for the difference in lymphocyte numbers in granuloma formation stems from differences in the host immune response between ectotherms and mammals. A similar assumption was made earlier by Cosma *et al.* (2003), who proposed that the recruitment of a greater number of lymphocytes in mammals might be the result of either an excessive and unnecessary immune response, or that the ectothermic immune systems might have evolved in a way to control the infection with fewer lymphocytes being required. This was further evaluated by Swaim *et al.* (2006) who took into account the fact that zebrafish possess a similar T-lymphocyte population to mammals, although the number in peripheral blood is lower when compared to mice and humans (Trede *et al.*, 2004). Furthermore, Swaim *et al.* (2006) showed that mutants lacking functional B and T lymphocytes (*rag1* mutant) died quicker than wild-type zebrafish, an observation also made in *rag1* deficient mice as well and demonstrating that adaptive immunity plays an important role in the protection

against mycobacteria (Swaim *et al.* 2006). The number of lymphocytes in granulomas of zebrafish infected with *M. marinum* NCIMB 1297 were found to be low in the current study, as previously noted for zebrafish infected with *M. marinum* M strain (Swaim *et al.* 2006) and for other ectothermic animals (Majeed *et al.* 1981; Talaat *et al.* 1998; Bouley *et al.* 2001; Cosma *et al.* 2003; Tobin and Ramakrishnan 2008). The finding that granulomas formed in humans following *M. marinum* infection possess a high lymphocyte number supports the possibility of an endothermic vs ectothermic difference rather than lymphocyte number being solely a *Mycobacterium* species-dependent feature.

3.4.5 Dystrophic calcification of mycobacterial granuloma

Detection of calcium deposits within granulomas, to indicate calcification, was examined using von Kossa staining of selected sagittal sections. Calcification, in general dystrophic calcification in tuberculous lesions is associated with the healing of the lesion in humans alongside fibrosis (Mohan 2007). The results from the current study showed no signs of calcification in the granulomas. However, only fish sampled at two months post-injection were available to stain with von Kossa due to the fact that most of the other fish were decalcified prior to processing. Investigation of this feature in granulomas of zebrafish has been previously described by Swaim *et al.* (2006), who also found no sign of calcification in zebrafish, even 4 months after infection. They therefore suggested that zebrafish were unable to achieve this activity. It has to be taken into consideration that the fish investigated in their study were fixed in Dietrich's fixative which has a light decalcifying effect due to glacial acetic acid being present in the mixture (Moore *et al.* 2002; Westerfield 2007; Kent *et al.* 2012). Other studies investigating calcification in mycobacterial lesions in other fish like goldfish, salmon or milkfish showed inconclusive results (Majeed *et al.* 1981; Bruno *et al.* 1998; Chang *et al.* 2006). Only one goldfish displayed evidence for calcification in some of the tubercles, visualised with Alizarin red staining (Majeed *et al.* 1981). Testing for

calcification within fish granulomas is not a common procedure like Ziehl-Neelsen and is therefore often not considered.

With respect to calcification as a feature of latent tubercle lesions in humans, it is important that a host model used to examine latent tuberculosis infections also displays this feature. In other model hosts, calcification of mycobacterial lesions is a rare feature. Most of the popular model host organisms like mice and rats do not display this feature in their lesions (Lenaerts *et al.* 2007; Dartois 2010; Singhal *et al.* 2011), whereas rabbits and guinea pigs possess this feature, but due to lack of immunological and molecular reagents these models are not ideal (Lenaerts *et al.* 2007; Dartois 2010). Non-human primates are so far the best model hosts for latent infection. Lin *et al.* (2009c) showed that cynomolgus macaques infected with *M. tuberculosis* develop a similar spectrum of granulomas including caseation, mineralization and fibrosis to the granuloma found in human active and latent disease (Capuano *et al.* 2003; Lin *et al.* 2009c). Despite being the ideal latent model from the disease progression point of view, the slow development of the disease in non-human primates leads to a requirement for a quicker modelling system.

3.4.6 Fibrosis of granulomatous lesions caused my *M. marinum*

Another feature signifying healing of lesions is fibrosis. This occurs as part of the healing succession during resolution of granulomatous lesions in humans, during which process fibroblasts accumulate around the periphery of the granulomas (Mornex *et al.* 1994). The reabsorption of the caseous material is accompanied by the deposition of collagen (Leung *et al.* 1999). Along with calcification, fibrosis is a key feature in latent human tuberculosis granuloma and similarly only rabbit, guinea pigs and human primates display this feature in their granulomas (Capuano *et al.* 2003; Lenaerts *et al.* 2007; Lin *et al.* 2009c; Dartois 2010). This similarity is not surprising as fibrosis is often followed by calcification as part of the natural healing process.

The results presented here indicated that fibrosis occurred during the later stages of the infection, with clear fibrosis observed by Masson's trichrome staining at 56 d.p.i. In fish, few publications are available in which the staining for fibrous connective tissue is part of the histological examination of granulomatous lesions. In agreement with their findings that zebrafish do not show any calcification, Swaim *et al.* (2006) detected no fibrosis in the granulomas they investigated, underlining their statement that zebrafish lesions do not heal. This disagrees with the recent findings from Parikka *et al.* (2012). This group showed that zebrafish lesions possess a fibrous or cellular cuff whose thickness increases over time. According to their research, most of the granulomas found in infected zebrafish were surrounded by a fibrotic and / or cellular cuff after 20 weeks of infection. It would be interesting to see whether similar lesions to the ones produced by *M. marinum* NCIMB 1297 (ATCC 927) in the Parikka publication also displayed signs of calcification. If so, the zebrafish *M. marinum* model would be a step closer to representing an accurate model system for latent human tuberculosis infections, especially with regard to granuloma formation.

3.5 *Conclusions*

In conclusion, the work presented in this chapter has focused upon granuloma formation in adult zebrafish over the first two months of *M. marinum* infection.

No granuloma formation was observed in fish infected with *M. marinum* NCIMB 1298. The non-pathogenic nature of this type strain was long hypothesised and widely accepted but had not yet been tested in adult zebrafish until now. Granuloma formation caused by the *M. marinum* strain NCIMB 1297 largely agreed with previously published observations in adult zebrafish infected with the *M. marinum* M strain but also extended the information available on fibrosis in *M. marinum* induced zebrafish granulomas.

The findings presented in this chapter indicate that zebrafish granulomas show a number of analogies with human tuberculosis granulomas such as necrosis, caseation

and fibrosis and this therefore supports the potential use of adult zebrafish as a model for active necrotising disease. Whether or not this model is applicable for chronic necrotising disease, as occurs in human latent tuberculosis infection, needs to be further investigated.

4 The innate immune response to *Mycobacterium marinum* infections in the kidney of adult zebrafish *in vitro and in vivo*

4.1 *Introduction*

The innate immune response is the primary defence mechanism of an organism against pathogens. Early recognition of pathogens and subsequent triggering of an appropriate response is the major function of innate immunity (van der Vaart *et al.* 2012). The response is non-specific and utilises evolutionarily conserved structures on pathogens termed PAMPs (pathogen-associated molecular patterns), which allow the host to respond within hours, in comparison to the adaptive response, which is more specific and needs longer for the response to fully develop (Abbas and Lichtman 2003; Lam *et al.* 2004; Trumstedt 2008; van der Vaart *et al.* 2012).

The PAMPs are recognized by pattern recognition receptors (PRRs), which are displayed on the surface of the cell, on endosomes and in the cytosol of macrophages, dendritic cells, endothelial cells, mucosal epithelial cells and lymphocytes (Abbas and Lichtman 2003; Stockhammer *et al.* 2009; van der Sar *et al.* 2009; Stockhammer *et al.* 2010; van der Vaart *et al.* 2012). Cell surface receptors include Toll-like receptors (TLRs) and scavenger receptors, while NOD-like receptors (NLRs) and RIG-like receptors (RLRs) can be found in the cytosol of the cell (van der Sar *et al.* 2009; Stockhammer *et al.* 2010; van der Vaart *et al.* 2012).

After recognition of the PAMPs through binding to the host PRRs, a pro-inflammatory and antimicrobial response is triggered to bacteria, which leads to the initiation of intracellular signalling pathways, resulting in the activation of gene expression and synthesis of molecules including pro-inflammatory *e.g*. TNFα, IL-1β

and IL-12 and chemotactic cytokines, as well as antimicrobial peptides (Lin *et al.* 2007; Stockhammer *et al.* 2009; 2010; van der Vaart *et al.* 2012).

The immune response to intracellular bacteria, such as *M. tuberculosis* or *M. marinum* follows the same mechanisms of recognition. After recognition of the PAMPs on the surface of the immune cells, transcription and translation of inflammatory cytokines occurs. Normally phagocytic cells like macrophages would start with the destruction of the invader by phagocytosis, but intracellular bacteria possess the ability to evade phagocytosis by manipulating the host cell and thus persist in the cells (van der Sar *et al.* 2009; van der Vaart *et al.* 2012). Consequently, additional mechanisms have to be enabled to destroy the pathogen. Innate immune responses might be able to limit bacterial growth for a certain period of time, but the adaptive immune response is required to control the infection and finally to eradicate the causative agent (van der Vaart *et al.* 2012). At this point, communication between the innate and the adaptive immune responses is crucial for successful elimination of the intracellular bacteria (van der Vaart *et al.* 2012). Communication between these two components of the immune system is mediated by cytokines. Two of the most important pro-inflammatory cytokines involved in this communication are Interleukin 12 (IL-12) and Interferon gamma (IFNγ) (Abbas and Lichtman 2003; van der Vaart *et al.* 2012).

Interleukin 12 is the principal mediator of the early immune response to intracellular microbes and is a key inducer of Th1 cell-mediated immunity, which provides a key component of the adaptive immune response to these microbes. The principal sources of IL-12 are activated mononuclear phagocytes and dendritic cells and secretion stimulates NK cells and T cells to produce IFNγ by promoting the differentiation of $CD4^+$ helper T lymphocytes into IFNγ producing Th1 cells (Abbas and Lichtman 2003; van der Vaart *et al.* 2012). IFNγ is another important cytokine in the immune response to intracellular bacteria, and both mediates the activation of macrophages and enhances the ability of the macrophage to kill intracellular bacteria

(van der Sar *et al.* 2009; van der Vaart *et al.* 2012). The production of IFNγ in turn stimulates the production of IL-12 and by doing so starts a positive amplification loop in the immune response (van Crevel *et al.* 2002; Abbas and Lichtman 2003; van der Sar *et al.* 2009). Due to the interaction between innate and adaptive immunity, any depletion of IFNγ or IL-12 can lead to insufficient eradication of the intracellular pathogen (Fig. 4-1) (Abbas and Lichtman 2003).

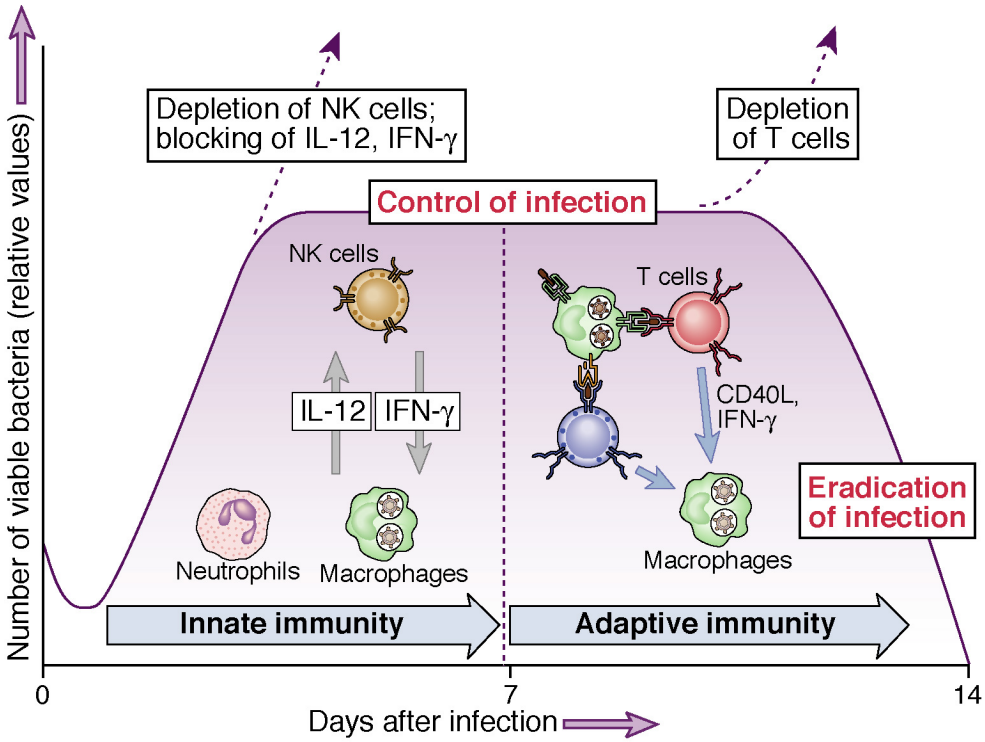

Figure 4-1: Innate and adaptive immunity to intracellular bacteria. Overview of the order of events in innate and adaptive immunity of mice infected with *Listeria monocytogenes* and the chronology of control and eradication of the intracellular bacteria. Taken from Abbas and Lichtman (2003)

As mentioned above, many intracellular bacteria develop mechanisms to evade destruction by phagocytic cells like macrophages (van der Vaart *et al.* 2012). Some species of bacteria are more developed than other species and are much more difficult to eradicate. Mycobacteria, such as *M. tuberculosis* and *M. marinum*, belong to this group of highly adapted intracellular bacteria, and it is known from work utilizing *M. tuberculosis* and *M. marinum* that they can manipulate both the innate and adaptive immune response, therefore delaying the host's immune response from the moment

they come into first contact with the bacterium (Shafiani *et al.* 2010; Shaler *et al.* 2012; van der Vaart *et al.* 2012).

Many of the components of the innate and adaptive immune responses have been conserved during the evolution of vertebrates, and therefore a variety of homologues can be found between fish and mammals. In zebrafish, with its fully sequenced genome, nearly all of the components of the innate and adaptive immune systems as well as the connecting pathways found in mammals, are displayed (Stockhammer *et al.* 2010; Wu *et al.* 2010; Ordas *et al.* 2011; van der Vaart *et al.* 2012).

Macrophages are the main phagocytic cell of the innate immune response, but they also participate in the adaptive immune response by processing and presenting antigens to lymphocytes (Wang *et al.* 1995; Abbas and Lichtman 2003; Joerink *et al.* 2006). The study of proliferation and differentiation of macrophages has been widely investigated in mammals, while knowledge on fish macrophages and leukocytes in general is less advanced (Neumann *et al.* 2000; Stafford *et al.* 2001; Joerink *et al.* 2006). Therefore, the development of established fish macrophage cell lines is essential to improve our understanding of the fish immune system and to examine their contribution in the host's immune defence mechanisms (Braun-Nesje *et al.* 1981; Stafford *et al.* 2001).

Cell culture systems using primary cells and cell lines are a very useful tool for research into the pathogenesis of disease. They allow basic questions on pathogenesis and host response to be investigated in a controlled *in vitro* environment (Bradford *et al.* 1994; Pandey 2013). Vertebrate cell lines are routinely applied in a wide range of research areas, from embryogenesis to oncology. Cell lines originating from different animal species and tissues not only provide a useful resource to study particular tissues, but they also allow the investigation of species-specific and cell-specific responses on a cellular level (He *et al.* 2006; Lakra *et al.* 2011).

Regarding non-mammalian *in vitro* culture systems, fish have been used as models for vertebrate development, genetics and immunology and the zebrafish in particular has come to the centre of attention in recent years (Collodi *et al.* 1992; Bradford *et al.* 1994)**.** Despite the successful introduction of zebrafish as a substitute or complementary model to those of mammals, the development and use of these *in vitro* cell culture systems are not as advanced as those for other vertebrate species (Collodi *et al.* 1992; He *et al.* 2006)**.**

The overall aim of this chapter was to investigate the innate immune response to *M. marinum* in the kidney of adult zebrafish *in vitro* and *in vivo*. Thus, an *in vitro* macrophage assay was developed and optimised and an *in vivo* infection model was developed to examine the expression of cytokine genes which are considered to be important in the innate immune response against tuberculosis. The basis for these investigations was the fact that pathogen-induced changes in host cells are generally accompanied by marked changes in gene expression due to pathogen-mediated reprogramming of the host's transcriptomic response during infection (Jenner and Young 2005; van der Sar *et al.* 2009). Changes in chemokine and cytokine gene expression are measurable through reverse transcription real-time polymerase chain reaction (RT-qPCR).

The specific objective aims of this chapter were:

(1) Development of an *in vitro M. marinum* infection model for adult zebrafish kidney macrophages by

   a. Optimising the isolation of macrophages from the kidney of zebrafish and developing a macrophage culture from individual zebrafish
   b. Infecting the zebrafish primary macrophage cultures with *M. marinum* NCIMB 1297 as a model *in vitro* for investigating infections, including

macrophage gene expression in response to the infection, analysed using real-time RT-qPCR.

(2) To determine if infection *in vivo* by intra-peritoneal injection (i.p.), the classical method for infecting zebrafish with *M. marinum* as well as other pathogens, is suitable in such small individuals. It is possible that the process of injection could, in itself, cause such a strong cytokine response that it might mask the true immune response to the pathogen.

(3) To test if the housekeeping genes commonly employed in zebrafish infection studies, Beta actin and Elongation factor 1 alpha (Elong 1a), were suitable for assessing gene expression in this infection model or whether an alternative candidate might be more promising.

(4) To investigate if there were differences in the innate immune response between *Mycobacterium* isolates by comparing gene expression of the pro-inflammatory cytokines IL-1β, TNFα and IL-12 in:

   a. *M. marinum* isolate NCIMB 1298 which is considered to be non-infective to zebrafish
   b. *M. marinum* isolate NCIMB 1297 which is known to be infective to zebrafish and which is commonly used in infection models

(5) To detect IFNγ gene expression and to determine:

   a. If both *M. marinum* strains trigger an inflammatory response and shift the immune response towards Th1 cells which express IFNγ
   b. The time point at which IFNγ becomes detectable

4.2 *Materials and Methods*

The innate, pro-inflammatory immune response to *M. marinum* infection in the kidney of adult zebrafish was investigated *in vitro* and *in vivo*.

4.2.1 Brown trout and zebrafish husbandry

Brown trout (*Salmo trutta*) used in this chapter were transported from Howietoun Fishery, Sauchieburn, Stirling, UK and maintained at 15°C in a flow-through system in the Aquatic Research Facility (ARF).

Zebrafish (*Danio rerio*) were raised and bred as described in Chapter 2.1.1. For the *in vivo* infection trials, the zebrafish were transferred from the tropical aquarium to the ARF, where they were acclimated to experimental conditions. Husbandry procedures performed during the infection experiments were as described in Chapter 2.1.1.

4.2.2 Preparation of bacteria

M. marinum strains NCIMB 1298 and NCIMB 1297 (ATCC 927) were used for the infection trials. Cultivation and preparation of the inoculation doses were performed as described in Chapter 2.2.1 and 2.2.2.

4.2.3 Infection of zebrafish macrophages with *M. marinum in vitro*: a preliminary optimisation study

The aim was to optimise macrophage isolation from zebrafish kidney and to subsequently develop an *in vitro* macrophage *M. marinum* infection assay. As zebrafish are so small, brown trout were used as a model to develop the best protocol for macrophage isolation and this method was subsequently optimised for the isolation of zebrafish kidney macrophages.

Three leukocyte isolation protocols based on density centrifugation with either Percoll® (Sigma-Aldrich Company Ltd, Dorset, UK) (Secombes, 1990; Frøystad et al.,

1998) or Histopaque® (Sigma-Aldrich) were used to determine which of these methods was best suited to separate leukocytes from the head kidney of brown trout.

4.2.3.1 Brown trout and zebrafish

Six small brown trout (~10 cm) were used. These were euthanized using Schedule 1 methods and the kidney was removed from the fish and placed into a 5 mL bijou containing 4.5 mL L-15 without glutamine (PAA, Somerset, UK) and to which 0.2% (v/v) heparin had been added (Sigma-Aldrich).

Fourteen zebrafish were used to compare the best protocol determined from the brown trout leukocyte isolation and 6 further zebrafish were used to compare this protocol with one involving isolation of leukocytes on a 37/54% Percoll gradient.

All fish utilised in this study were euthanised using terminal anaesthesia with 10% benzocaine (w/v) (Sigma-Aldrich).

4.2.3.2 Preparation of 34/51% and 37/54% Percoll® gradients

The Percoll gradient was diluted from the original stock using distilled water (dH_2O) and Hanks Buffered Saline Solution (HBSS) (Sigma-Aldrich), as shown in Table 4-1 following the original protocol of Secombes (1990) and Frøystad *et al.* (1998) for the 34/51% and 37/54% gradients, respectively.

Table 4-1: Percoll gradients used for isolating brown trout macrophages

	Percoll gradient concentrations			
Volume (mL)	**34%**	**37%**	**51%**	**54%**
Percoll	3.4	3.7	5.1	5.4
L-15	-	-	-	4.6
Hanks Buffered Saline Solution	0.7	6.3	1.0	-
Distilled water	5.9		3.9	-
Total volume	**10**	**10**	**10**	**10**

4.2.3.3 Leukocyte isolation

For brown trout, kidneys were individually homogenised though a 40 µm cell strainer (BD Bioscience, Oxford, UK) into a 55 mm Petri dish (Sterilin Ltd, Newport, UK) and 1.5 mL of the cell suspension was layered onto a) Histopaque, b) 34/51% Percoll and c) 37/54% Percoll gradients in 5 mL collection tubes (BD Bioscience).

Single or pooled zebrafish kidneys were homogenised in a similar way, using different volumes of L-15 according to Table 4-2 to determine the most suitable volume of L-15 medium and Histopaque to use to isolate leukocytes. In addition, the isolation of zebrafish kidney leukocytes with Histopaque was compared to isolation with 37/54% Percoll gradient.

Table 4-2: Experimental set up for optimisation of mononuclear cell isolation from single and pooled zebrafish kidneys using Histopaque

	Kidney (single or pooled)	L-15 medium with 0.2% heparin (mL)	Histopaque (mL)
15mL falcon tube	Pooled	5.0	5.0
5 mL collection tube	Pooled	2.5	2.5
5 mL collection tube	Single	2.5	2.5
5 mL collection tube	Single	2.0	2.0
5 mL collection tube	Single	1.5	1.5

A summary of the work flow for this study is shown in Fig. 4-2. The suspensions were centrifuged at 15°C for the brown trout cells and 28°C for the zebrafish cells in a Sigma 4K15 laboratory centrifuge (Sigma-Aldrich). Cells layered onto Histopaque and 34/51% Percoll gradient were centrifuged at 400 x g for 35 min, while the 37/54% Percoll gradient was centrifuged at 600 x g for 40 min.

After terminal anaesthesia, kidney was removed and placed in L-15 containing 0.2% Heparin

Kidney was homogenised through a 40 µm cell strainer and kidney suspensions were layered on:
a) Histopaque
b) 34/51% Percoll
c) 37/54% Percoll

The suspensions were centrifuged for:
a+b) 35 min at 400 x g at 15°C
c) 40 min at 600 x g at 15°C

Collected mononuclear cells were washed and re-suspended in L-15 containing 0.01% Pen Strep (10000 units mL^{-1})
Duplicates of leukocyte suspension were transferred into a Nunc® Lab-Tek® Chamber Slide™ system

Figure 4-2: Workflow for leukocyte isolation from brown trout and zebrafish kidneys using Histopaque or 34/51% Percoll or 37/54% Percoll gradients.

Following centrifugation, the band containing the leukocytes was isolated with a sterile 1 mL pipette and transferred to a new tube. Isolated cells from the Histopaque and 34/51% Percoll gradient were washed with L-15 containing 0.01% Penicillin Streptomycin (PenStrep, 10000 units mL^{-1}, Invitrogen, Paisley, UK) and centrifuged at 800 x g for 7 min at 15°C or 28°C. Cells isolated from the 37/54% Percoll gradient were washed with HBSS and centrifuged at 400 x g for 10 min at 15°C or 28°C.

After washing, the supernatant was removed and the cell pellet was re-suspended in L-15 containing PenStrep, cell pellets comprising brown trout leukocytes were re-suspended in 500 µL of L-15 medium resulting in a final volume of 550 µL leukocyte suspension while zebrafish leukocyte pellets were re-suspended in 300 µL.

Two hundred and fifty microliters of brown trout kidney cells or 150 µL of the isolated zebrafish kidney cells were placed into 8 well Nunc® Lab-Tek® Chamber Slide™ systems, using two chambers for each cell separation method. Chamber slides were incubated at 15°C (Brown trout cells) and 28°C (zebrafish cells) for 2.5 h to allow the macrophages to attach, then, non-attached cells were removed by washing the wells with L-15 containing PenStrep. After a further 2 h incubation, the washing procedure was repeated before cells were incubated in L-15 containing PenStrep overnight. All solutions were kept at the same temperature as the incubated cells to avoid cell detachment.

After the overnight incubation, the cells on the Nunc chamber slides were washed again with fresh L-15 medium to remove cells which had detached overnight. The number of remaining attached cells was determined by removing the culture medium from the chambers and adding 50 µL lysis buffer (0.1 M citric acid, 1% (v/v) Tween 20, 0.05% (w/v) crystal violet) to the cells (Secombes 1990). After a 2 min incubation, the released nuclei were counted using a haemocytometer. Numbers of attached cells were calculated using Equation 1 after counting the four large squares under 10 x magnification.

Number of attached cells per ml = $\left(\frac{\text{counted nuclei} \times 10^4}{\text{number of squared}}\right) \times 0.05$ *(Equation 1)*

4.2.3.4 Examination and characterisation of adherent leukocyte population

The identity of attached cells was investigated by placing 50 µL of the remaining leukocyte suspensions onto a microscope slide within a circle marked with a PAP pen. After 2.5 h incubation in a moist chamber, the microscope slides were washed with L-15 and the remaining attached cells were fixed with ice-cold, absolute methanol and left to air dry before staining using a Rapid Romanowski staining kit (Raymond A. Lamp, Eastbourne, UK). Adherent leukocyte populations were then

examined under an Olympus light microscope BX51 and images obtained using AxioVision image capture software Version 4.0 (AxioVs40 V 4.8.2.0) (ZEISS, Jena, Germany) as described in Chapter 3.2.6., to determine the abundance of mature macrophages.

4.2.3.5 Infection of the *in vitro* culture with *M. marinum*

Leukocytes were isolated using Histopaque from individual kidneys of 24 zebrafish, as described in Section 4.2.3.3., with each resulting cell pellet individually re-suspended in 700 µL of L-15 medium supplemented with antibiotics. One hundred microliters of the re-suspended cells were transferred into a 96 well plate, seven replicates per sample. After overnight incubation and a final washing step, half of the macrophage cultures were infected with 20 µL of 10^4 cfu mL^{-1} *M. marinum* NCIMB 1297 while 20 µL of sterile PBS were added to the other half as a negative control. The macrophages were maintained at 28°C and sampled at different time points following infection with mycobacteria.

Three *in vitro* infection studies were performed, each comprising kidneys from 8 fish: In the first experiment, cells were sampled at 0, 1, 2 and 4 h.p.i.; in the second and third experiments the cells were counted at 0, 1, 3 and 6 h.p.i. In the first two experiments only the attached leukocytes were counted, while in the third experiment RNA was also extracted from samples to investigate gene expression. The attached cells in experiment 3 were removed from the culture plate by carefully scratching the bottom of the wells with a sterile 10 µL pipette tip to release the cells at 0, 1, 3 and 6 h.p.i. The cells were subsequently transferred to RNase-free 0.5 mL microcentrifuge tubes and the culture medium was removed by centrifuging the suspension at 1000 x g for 7 min. The supernatant was carefully discarded and the cell pellet was re-suspended in 400 µL TriReagent (Sigma-Aldrich) and stored in -80°C. RNA extraction and cDNA synthesis was performed as described in Section 4.2.4.5. Samples were pooled according to treatment and sampling time to gain enough material to perform

PCR and RT-qPCR as described in Section 4.2.4.6 and 4.2.4.7. The expression of immune-related genes Interleukin 1 beta (IL-1β), Tumor necrosis factor alpha (TNFα) and Toll-like receptor 9 (TLR9) was investigated using Beta actin as housekeeping gene. Estimation of the number of attached leukocytes in the wells of the three experiments was performed as described in Section 4.3.2.3.

4.2.4 Infection of zebrafish with *M. marinum in vivo*

4.2.4.1 Experimental design of the preliminary optimisation study

To optimise experimental conditions for the main study, 54 zebrafish were injected i.p. with either sterile 1x phosphate buffered saline (PBS) or *M. marinum* isolate NCIMB 1297, as shown in Table 4-3 and described in Chapter 2.1.2 and 2.2. Fish were sacrificed and the kidney was sampled on 0, 1, 2, 3 and 7 days post injection (d.p.i.) as described in Chapter 2.2.3. The RNA was extracted from each individual kidney and cDNA was subsequently synthesised, as described in Section 4.2.6.

4.2.4.2 Experimental design of the main study

To examine the pro-inflammatory innate immune response to the two mycobacteria isolates *M. marinum* NCIMB 1297 and NCIMB 1298, a total number of 124 zebrafish were utilised. 90 zebrafish were injected i.p. with either sterile 1x PBS, *M. marinum* isolate NCIMB 1297 or *M. marinum* isolate 1298 as described in Table 4-4. A fourth group of 34 uninjected (control) zebrafish was also included. Fish were sacrificed and kidneys sampled on 0, 1, 3, 5, 7, 11 and 14 d.p.i. Fish sacrificed in this experiment were obtained from the same infection experiment as the fish in Chapter 3 to obtain comparable results. For a more detailed analysis, fish were also sampled at 1, 3, 5 d.p.i. to include additional time points.

Table 4-3: Experimental setup for initial optimisation study where zebrafish were injected with either PBS or *M. marinum* NCIMB 1297

Sampling days (d.p.i.)	Number of Zebrafish	
	Control (Mock infected, PBS)	Infected with *M. marinum* strain NCIMB 1297
0	6	0
1	6	6
2	6	6
3	6	6
7	6	6
Total fish number	**30**	**24**

Table 4-4: Experimental setup for the main study investigating the pro-inflammatory immune response of zebrafish to *M. marinum* by injection of zebrafish with PBS (control), *M. marinum* NCIMB 1297 or *M. marinum* NCIMB 1298. Additionally, a non-injected control was included.

Sampling day (d.p.i.)	Number of Zebrafish			
	Non-injected	PBS (mock) injected	Infected with *M. marinum* isolate NCIMB 1298	Infected with *M. marinum* isolate NCIMB 1297
0	4			
1	5	5	5	5
3	5	5	5	5
5	5	5	5	5
7	5	5	5	5
11	5	5	5	5
14	5	5	5	5
Total fish number	**34**	**30**	**30**	**30**

4.2.4.3 Organ dissection

Dissection of the kidney was performed as described in Chapter 2.3.2.

4.2.4.4 DNA isolation

DNA was extracted from fish belonging to the main study (Section 4.2.4.2) to establish that the fish were infected with the bacteria, and were sampled at 5 d.p.i using the Nucleospin Tissue Kit as described in Chapter 2.5.1. The liver, stomach and

reproductive tract rather than kidney and spleen were used for DNA extraction due to the fact that the kidney had already been used for the RNA extraction.

4.2.4.5 RNA extraction and cDNA synthesis

For the optimisation of RNA extraction, RNA was extracted from sampled kidneys using two different methods: (1) Qiagen RNeasy column method using the RNeasy Mini kit as described in Chapter 2.5.3 and (2) TriReagent method with subsequent modifications (Chapter 2.5.2). Modifications to the manufacturer's protocol included (i) an extension of the isopropanol precipitation step which was replaced by overnight incubation at -20°C and (ii) the addition of an RNA carrier in the form of linear polyacrylamide (LPA). The optimised TriReagent method was applied in the main experiment as well as the extraction of RNA from the cell culture experiment as described in Chapter 2.5.2. Isolated RNA was subsequently converted to cDNA using a high capacity kit, as described in Chapter 2.5.5.

4.2.4.6 Polymerase Chain Reaction (PCR)

The extracted DNA from the cell culture experiment and the DNA extracted from the internal organs at 5 d.p.i. of the main study were analysed for the presence of *Mycobacterium* spp. using the polymerase chain reaction (PCR), as described in Chapter 2.5.6. The primers Tb11 (5′-ACCAACGATGGTGTGTCCAT-3') and Tb12 (5′-CTTGTCGAACCGCATACCCT-3') were used for detecting *Mycobacterium* spp. in the DNA samples as this primer pair amplified a 441 bp fragment of the heat shock protein 65 gene (hsp65 gene) in the *Mycobacterium* genome (Telenti *et al.* 1993).

Optimisation of PCR conditions for the chosen primer sets was carried out using the cDNA from the preliminary study. Accuracy and optimal annealing temperature for primers for housekeeping genes and genes of interest were tested prior to using them for the RT-qPCR), with results being summarised in Table 4-5. The optimal annealing temperature (Ta) for each primer set was calculated by using the

natural logarithm (ln) of the product size and the melting temperature (Tm) of the smaller primer, which was given by the MWG order sheet, following the formula:

$$Ta = \ln(\textit{product size}) + Tm(\textit{smaller primer})$$

A gradient PCR was performed for each primer set using the Ta as the mid value of a 5°C gradient to confirm the accuracy. Results of the PCR optimisation are not included in this Chapter.

4.2.4.7 Real-time quantitative PCR (qPCR)

Gene expression analysis was performed using SYBR green RT-qPCR, as described in Chapter 2.5.8. Due to the number of samples obtained from the main study, only 1, 3, 7 and 14 d.p.i. were analysed in this Chapter to measure the expression of the immune genes IL-1β, TNFα, Interleukin 12 (IL-12) and Interferon gamma (IFNγ) as summarised and described in Table 4-5.

Validation of the housekeeping genes Beta actin, Elong 1a and ribosomal protein L13a (RPL13a) was performed by geNorm and Normfinder which are included in the GENEX software (v.2.6.4; www.multiD.se). In all cases GENEX was used for normalisation and analysis of the results. Gene expression data were efficiency corrected, normalised to the best suitable housekeeping genes and displayed relative to the control groups (non-injected and PBS-injected fish) in a log2 scale.

4.2.4.8 Validation of housekeeping genes

Validation of the three housekeeping genes Beta actin, Elong 1a and RPL13a was performed in two different ways; (i) by geNorm and (ii) by Normfinder.

GeNorm is an algorithm to determine the stability value (M-value) of chosen housekeeping gene candidates (Vandesompele *et al.* 2002). Normfinder is freely available software for identifying an optimal normalisation gene or applying an algorithm on a given set of candidate genes and subsequently ranking them according to a calculated expression stability value (Andersen *et al.* 2004). In contrast to

geNorm, Normfinder includes inter- and intra-group variation in its algorithms to determine the stability values. For both programs, the lower the resulting stability value is, the more suitable the housekeeping candidate is.

4.2.5 Statistical analysis

Statistical analysis of the RNA extraction was performed utilizing GraphPad Prism software. The non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test was applied to test for significant differences in the amount of extracted RNA. Statistical significances in immune gene expression were also calculated with GraphPad Prism. Determination of significant differences within a sampling day was performed by assessing the non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test on the ∆ct values which relate to the corrected efficiency and to the housekeeping genes' normalised ct values. One-way ANOVA was not applicable due to the significant outcome of the Levene's test indicating that the variances of the sample groups displayed significant inhomogeneity.

Significant differences for each treatment across the sampling days were estimated in the same way. The Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test was applied to the ΔΔct values which comprised the Δct value relatively expressed in a log2 scale to the control groups. Utilising the ΔΔct values instead of the Δct values minimised inter-plate variations in the experiment

Table 4-5: Primer sets for PCR and qPCR including their target gene name and gene symbol, primer sequence, expected product size and additional references

Gene name (Gene Symbol)	Primer set	Sequence	Product size	Reference
Elongation factor 1 α (Elong 1a)	Elong F2 Elong R2	5'-TGCCTTCGTCCCAATTTCAG-3' 5'-TACCCTCCTTGCGCTCAATC-3'	101	(Wang *et al.* 2011)
Ribosomal protein L13 a (RPL13a)	RPL F RPL R	5'-TCTGGAGGACTGTAAGAGGTATGC-3' 5'- AGACGCACAATCTTGAGAGCAG-3'	148	(Tang *et al.* 2007)
Beta actin	Ba F Ba R	5'-CGAGCAGGAGATGGGAACC-3' 5'-CAACGGAAACGCTCATTGC-3'	104	(Rodriguez *et al.* 2008)
Interleukin1 β (IL-1β)	IL1 F2 IL1 R2	5'-CCACGTCTCCACATCTCGTA-3' 5'-CCTGTCCATCTCCACCATCT-3'	200	(Rodriguez *et al.* 2008)
Tumor necrosis factor α (TNFα)	TNF F3 TNF R3	5'-ACCAGGCCTTTTCTTCAGGT-3' 5'-TGCCCAGTCTGTCTCCTTCT-3'	303	(Rodriguez *et al.* 2008)
Toll-like receptor 9 (TLR9)	TLR 9F TLR 9R	5'-GGGGAAGATGGCGCTTTCAG-3' 5'-CCAGAAGAGCGGCTGCACTC-3'	332	(Meijer *et al.* 2004)
Interleukin 12 (IL-12)	IL12 F3 IL12 R3	5'-GCATGGCTCTGGCTCTGGCTC-3' 5'-AGTTAGCGCTGACGGACGGC-3'	234	Primer3 (Untergasser *et al.* 2012)
Interferon γ (IFNγ)	IFN VoiF IFN VoiR	CTTTCCAGGCAAGAGTGCAGA TCAGCTCAAACAAAGCCTTTCG	101	(Vojtech *et al.* 2009)

4.3 *Results*

4.3.1 Development and optimisation of an *in vitro M. marinum* infection assay

Brown trout was used as a model to develop the best protocol for macrophage isolation and then this method was subsequently used with zebrafish to develop an *M. marinum in vitro* infection assay.

4.3.1.1 Determination of the most suitable leukocyte isolation method from brown trout and zebrafish kidney using Percoll® and Histopaque® gradients

Success of isolation of head kidney leukocytes from brown trout was compared using Histopaque, 34/51% Percoll or 37/54% Percoll gradients. The efficiency of each of the three gradients for leukocyte isolation was assessed by examination of the band comprising leukocytes which was formed following centrifugation, as shown in Fig. 4-3.

In the case of the Histopaque separation method, this layer was clearly visible at the intersection between the Histopaque layer and the L-15 layer (Fig. 4-3A). At the bottom of the collection tube, a red cell pellet was visible which contained the erythrocytes. Leukocyte isolation using Histopaque resulted in a mean of $4.1 \times 10^4 \pm 7.2 \times 10^3$ attached cells mL^{-1} for brown trout (Fig. 4-4).

Examination of the band following centrifugation on the 34/51% Percoll gradient showed a black-reddish band at the interphase between L-15 medium and Percoll. It appeared that this band was an accumulation of erythrocytes and other kidney tissue components as well as leukocytes. The same phenomenon was observed for the 37/54% Percoll gradient (Fig. 4-3 B+C).

The 34/51% isolation methods resulted in slightly fewer cells ($3.0 \times 10^4 \pm 3.8 \times 10^3$ cells mL^{-1}) than with Histopaque while the 37/54% gradient method resulted in a mean cell number of $5.5 \times 10^4 \pm 1.2 \times 10^3$ mL^{-1}. No significant

differences were detectable in the number of attached cells between the three methods (Fig 4-4) (p=0.505).

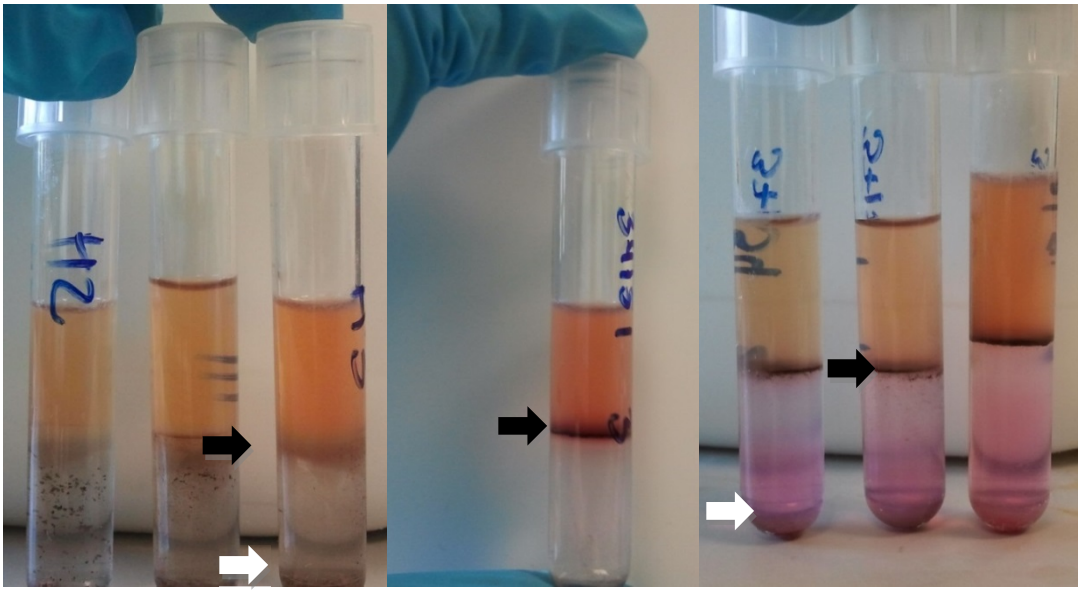

Figure 4-3: Isolation of brown trout kidney macrophage cells using gradient centrifugation using A) Histopaque, B) 34/51% Percoll or C) 37/54% Percoll. Black arrows indicate the leukocyte containing layer at the interface between Histopaque or Percoll and L-15 medium. White arrows show the erythrocyte pellet.

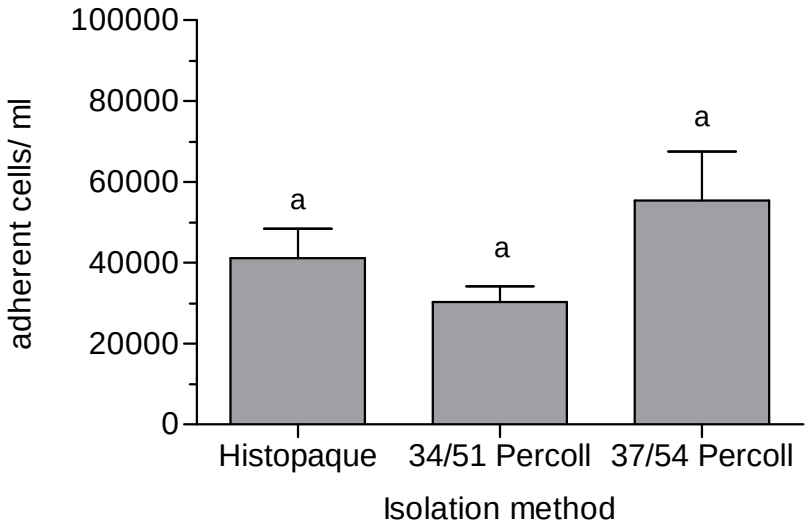

Figure 4-4: Mean number of attached cells per mL (mean ± SE) counted for each of the three treatments, Histopaque, 34/51% Percoll and 37/54% Percoll. Cells were isolated and cultured individually from six brown trout kidneys. Adherent cell numbers were estimated after overnight incubation by applying lysis buffer and counting released nuclei. Significant differences were determined using a non-parametric Kruskal-Wallis test.

For the isolation of the leukocytes from pooled or individual zebrafish kidneys, homogenisation in 2 mL or 1.5 mL L-15 medium and subsequently layering onto equal

volume of Histopaque, gave the best results, with the latter resulting in a distinct cell layer. Comparison of the Histopaque and the 37/54% Percoll cell separation methods showed no significant differences in the number of zebrafish kidney leukocytes isolated. As expected, the cell numbers obtained from the zebrafish kidney leukocyte cultures were lower compared to the numbers from the brown trout cell cultures. On average, a leukocyte culture from an individual zebrafish comprised $1.5 \times 10^3 \pm 1.6 \times 10^2$ cells per mL when cells were isolated on Histopaque, while the 37/54% Percoll gradient method yielded $2.0 \times 10^3 \pm 4.10 \times 10^2$ cells per mL (Fig. 4-5). No significant differences were detectable between the two treatments (p=0.770).

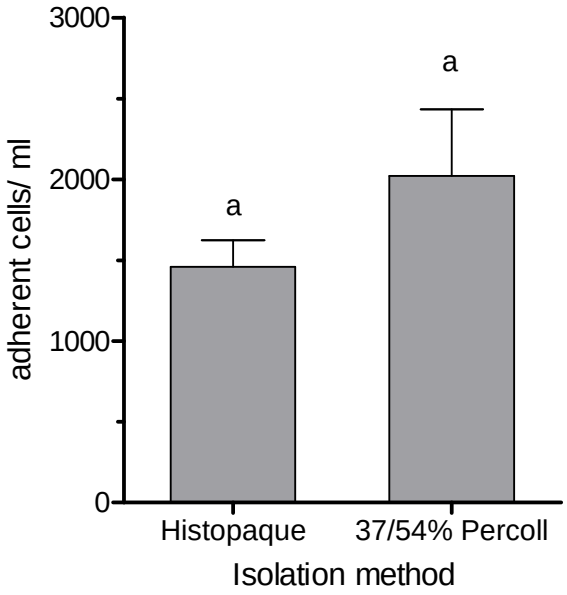

Figure 4-5: Mean number of adherent cells per mL (mean ± SE) counted for each of the two treatments, Histopaque and 37/54% Percoll. Cells were isolated and cultured individually from six zebrafish kidneys. Adherent cell numbers were estimated after overnight incubated by applying lysis buffer and counting of released nuclei. Significant differences were determined using a non-parametric Kruskal-Wallis test.

Comparison of microscope slides covered with the different lymphocyte suspensions revealed that despite repetitive washing of the attached cells, a large number of lymphocyte-like cells were still attached to the slides as well as the desired monocytes and macrophages. This was observed on all slides independent of the fish species used or the cell isolation method applied. Examples of the attached cell populations on slides from the Histopaque separation method are shown in Fig. 4-6.

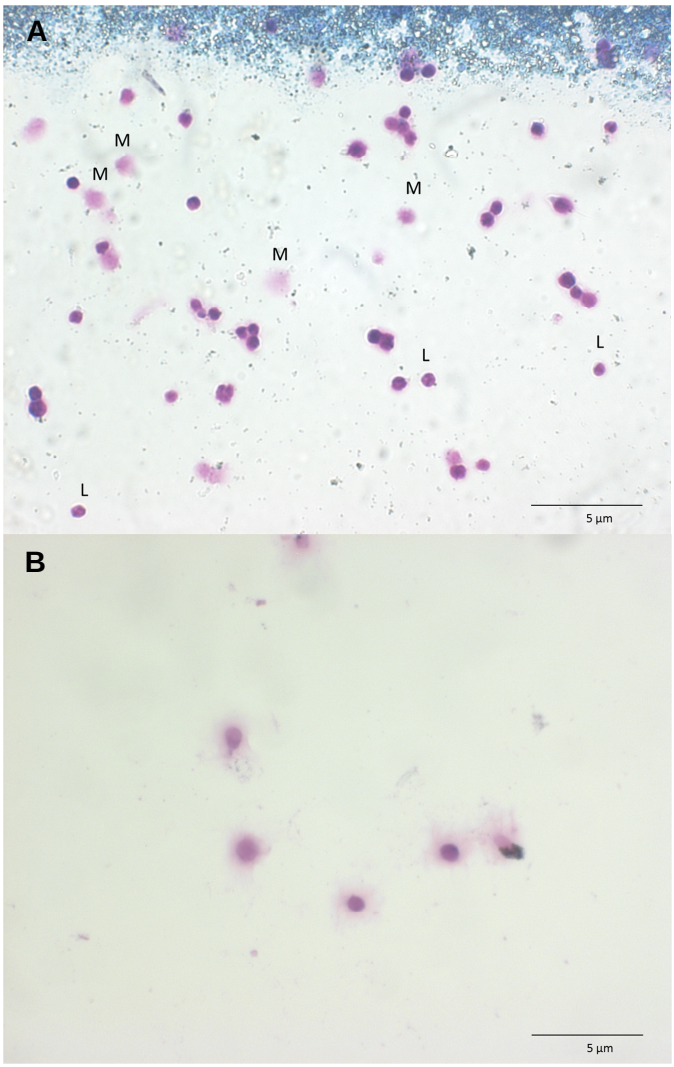

Figure 4-6: Adherent cell populations obtained following isolation of brown trout kidney cell population on Histopaque including A) a mix of leukocytes (M macrophages, L lymphocytes) and B) macrophages. The leukocyte suspension was allowed to attach to microscope slides for 2.5 hours. Non-attached cell were removed by washing with L-15 and the remaining adhered cells were stained with rapid Romanowski stain. Photographs were taken using 40x magnificantion.

Lymphocytes were distinguished by their small size and dense purple nuclei while monocytes were slightly larger and had smaller nuclei compared to the lymphocytes. Macrophages were recognisable by their large size and pink cytoplasm. As the cultures comprised a mixture of cells, rather than only macrophages, they were referred to as leukocyte cultures.

4.3.1.2 Development of a *M. marinum in vitro* infection assay using leukocyte cultures

After overnight incubation of the leukocyte cultures isolated on Histopaque from zebrafish kidneys, the medium in the wells was removed and the remaining attached leukocytes carefully washed with fresh medium. Half of the wells were infected with *M. marinum* NCIMB 1297 while the other half were mock-infected with PBS. The number of attached cells was estimated by removing the medium at 0, 1, 3, 4 and 6 h.p.i. and lysis of the remaining cells from the well with subsequent counting of the nuclei. The cell counts obtained from the different wells are shown in Table 4-6 and summarised as %.cell counts.

Table 4-6: Cell counts (as percentages) of three times two replicates of adherent zebrafish kidney leukocytes maintained *in vitro* over time in a 96 well plate after inoculation with *M. marinum* NCIMB 1297 compared to control wells (PBS inoculated). The cell number in the control was set as 100% for each time point and the % of cells counted in the infected wells were calculated in respect to the control wells.

	Cell counts (%)					
	0 hpi	**1 hpi**	**2 hpi**	**3 hpi**	**4 hpi**	**6 hpi**
Experiment 1	100.0	79.1	84.2	-	76.9	-
Experiment 2	100.0	81.0	-	78.3	-	38.1
Experiment 3	100.0	96.3	-	78.2	-	75.0
Mean	100.0	85.5	84.2	78.2	76.9	56.5
Standard Deviation	0.0	9.4		0.1		26.1

hpi – hours post-infection

At 1 h.p.i., the mean percentage of adherent cells was 85.5 ± 9.4% (mean ±SD) and this further decreased to 56.5 ± 26.1% at 6 h.p.i. The control wells, which were mock-infected with PBS, looked healthy during the entire experiment.

Due to the low replicate number in this preliminary experiment, no statistical test was applicable but the data and the decreasing number of cells in the infected wells relative to controls, indicated that the infection of the leukocytes with *M. marinum* NCIMB 1297 was successful and the assay was then used to investigate gene expression following *M. marinum* infection.

4.3.1.3 RNA extraction and gene expression of infected macrophages *in vitro*

RNA was extracted from seven replicates of both infected and mock-infected wells at 1, 3, and 6 h.p.i. using TriReagent with RNA yields ranging from 27.39 ± 7.98 to 51.36 ± 12.73 ng μL^{-1} and no significant differences in RNA yields detectable between the different time points or between the two treatments (p=0.871).

Successful infection of the cell cultures with *M. marinum* NCIMB1297 was confirmed by the amplification of the 65ka heat shock protein (441bp) in the genome of *M. marinum* by PCR in the infected wells (Fig. 4-7).

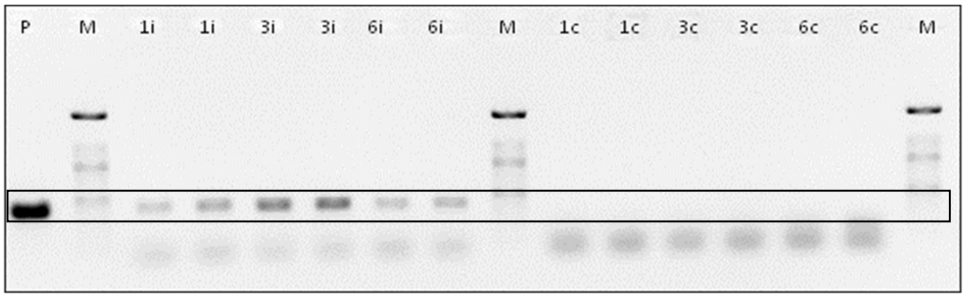

Figure 4-7: Gel electrophoresis on a 1.5% agarose gel of DNA extracted leukocyte cell culture at 1, 3 and 5 h.p.i. experimentally infected (i) with *M. marinum* NCIMB 1297 (1i, 3i, 6i) and PBS (1c, 3c, 6c) as contron (c) including 100 bp ladder as gel marker (M) and positive control (P, *M. marinum* NCIMB 1297 DNA). The frame encloses the area of expected 65kDa mycobacterial heat shock-protein (441bp).

To investigate changes in the immune response of infected leukocytes in comparison to non-infected cells, the gene expression of IL-1β, TNFα and TLR9 was measured at 1, 3, and 6 h.p.i. by real-time PCR. In addition, the expression of beta-actin was measured as a housekeeping gene. Fig. 4-8 summarises the expression of the three investigated genes after normalisation to the housekeeping gene.

The IL-1β expression was significantly up-regulated in infected cells compared to the control cells at 1 h.p.i. (7.942 ± 0.9926, p = 0.0286). At 3 h.p.i., expression of this gene was hardly detectable (0.2129 ± 0.9158) and not significantly different to the control group. After another 3 hours, the expression of IL-1β appeared to be down-regulated (-4.395 ± 5.300) although this did not appear to be significantly different from

the control level at 6 h.p.i. The standard error was, however, quite large at 6 h.p.i. compared to the other two sampling points.

The TNFα expression in the infected cells was very low (ranging from -0.1562 ± 0.1758 at 1 h.p.i. and 0.09525 ± 0.1693 at 3 h.p.i) and not significantly different to the control cells at these time points. The increase in expression of TNFα across the sampling days, from 1 to 6 h.p.i (0.9521 ± 0.1693) was significant ($p = 0.0183$).

Gene expression of TLR9 in infected cells was significantly up-regulated compared to the control cells at 1 and 3 h.p.i. (7.910 ± 1.029 and 3.533 ±0.6846, $p = 0.0286$). At 6 h.p.i., the average expression of TLR9 was still positive but revealed a large standard error (1.708 ± 7.944). No significant differences were detectable between the three sampling points.

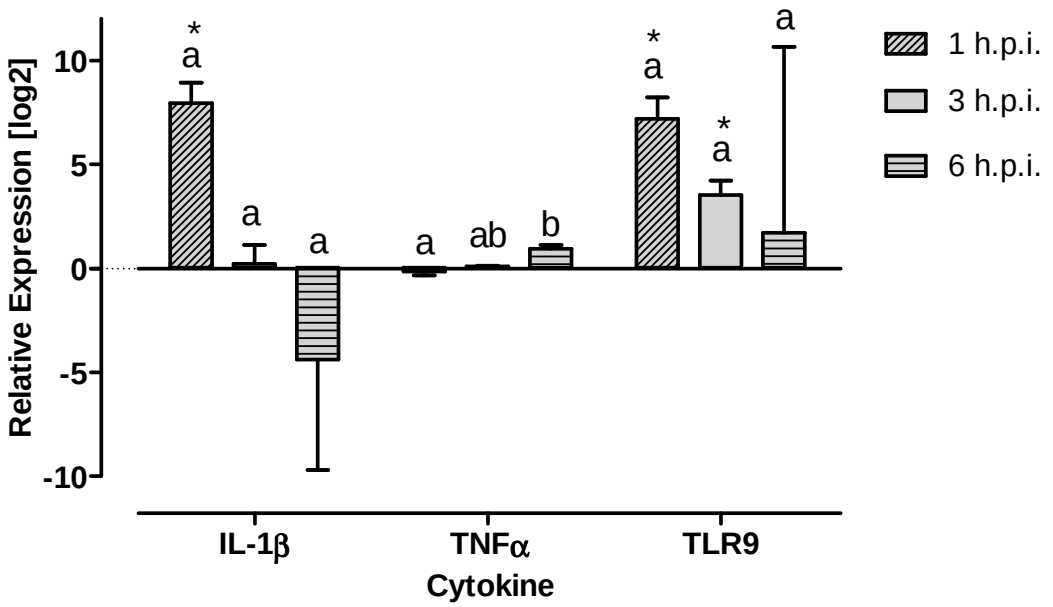

Figure 4-8: Kinetics of expression of immune genes interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα) and toll like receptor 9 (TLR9) in cultured zebrafish kidney leukocytes experimentally infected with *M. marinum* NCIMB 1297 and sampled at 1, 3, and 6 hours post-infection (h.p.i). Data represent the relative expression (in log2 scale) including the mean value and standard error compared to PBS-infected control cells at the same time points and normalised to the housekeeping gene Beta actin (n=4). Statistically significant differences ($p<0.05$) to control cells have been determined by a non-parametric Mann-Whitney test and are indicated by an asterisk (*). Statistically significant differences ($p<0.05$) between sampling points have been determined by a non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test and are indicated by different letters above data sets.

4.3.2 Infection of adult zebrafish with *M. marinum in vivo*

An *in vivo* infection model was optimised to examine the expression of the cytokine genes which are considered to be important in the innate immune response against tuberculosis in humans.

4.3.2.1 RNA extraction from individual zebrafish kidneys

RNA extraction from individual zebrafish kidneys was performed using Qiagen RNeasy columns and the TriReagent extraction method. The Qiagen method resulted in a mean RNA yield of 342.1 ± 16.70 ng μl^{-1} (Fig. 4-9). For the TriReagent extraction method (trizol (normal)), the resulting RNA yield was significantly lower ($p<0.001$) and revealed an average value of 18.64 ± 2.02 ng μl^{-1} following the manufacturer's protocol.

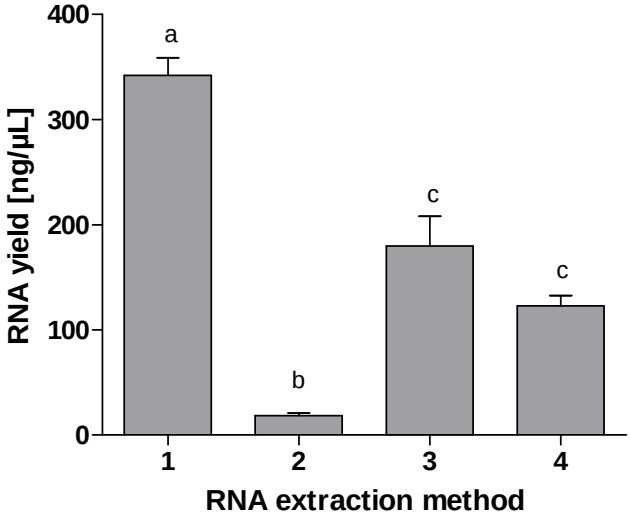

Figure 4-9: Comparison of RNA yield (mean + SEM) from samples extracted from individual zebrafish kidneys using (1) Qiagen RNeasy columns (RNeasy column) and (2) TriReagent (trizol). The TriReagent method was optimised by (3) overnight precipitation at -20°C (trizol + ONP -20) and (4) addition of an RNA carrier in form of linear polyacrylamide (LPA) (trizol + LPA + ONP -20). Significant differences were determined by the non-parametric Kruskal-Wallis test with subsequent Dunn's multicomparison *post hoc* test with significant differences indicated by different letters a-c.

Optimisation of the standard protocol by an extended precipitation step overnight at -20°C (trizol + ONP -20) significantly increased the yield of extracted RNA yield to a value of 180.0 ± 27.98 ng μl^{-1} ($p<0.001$). The addition of linear

polyacrylamide (LPA) as an RNA carrier to the extended precipitation step did not show any significant changes in the resulting RNA yields of 122.8 ± 9.82 ng μl^{-1}.

4.3.2.2 Confirmation of successful infection

Confirmation of the successful infection of the zebrafish with *M. marinum* was established by PCR. The DNA extracted from the internal organs (liver, stomach and reproductive organs) at 5.d.p.i. from zebrafish experimentally injected with PBS, *M. marinum* NCIMB 1297 and NCIMB 1298 was tested for the presence of the 65 kDa mycobacterial heat shock-protein. In Fig. 4-10 the results of the PCR are shown through visualisation of the PCR products on an agarose gel.

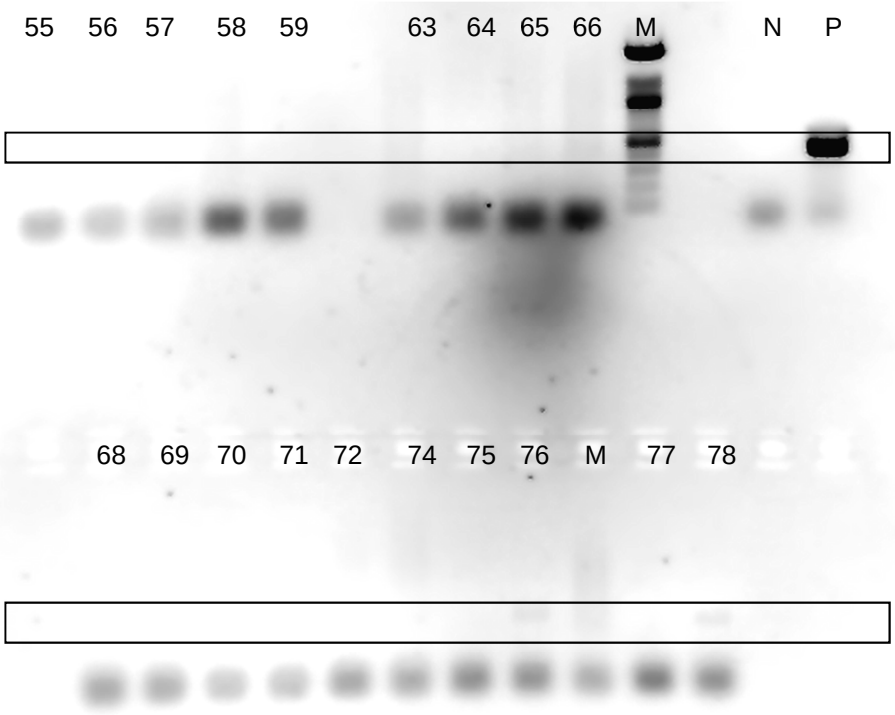

Figure 4-10: Gel electrophoresis on a 1.5% agarose gel of DNA extracted from internal organs (liver, stomach and reproductive organs) at 5 d.p.i. for zebrafish experimentally injected with *M. marinum* NCIMB 1297 (74-78), NCIMB 1298 (68-72) ,PBS (63-66) and non-injected fish (55-59) including 100 bp ladder as gel marker (M). Negative control (N, dH_2O) and positive control (P, *M. marinum* NCIMB 1297 DNA) were included. The frame encloses the area of expected 65 kDa mycobacterial heat shock-protein (441 bp).

No mycobacteria were detectable in the fish sampled from the non-injected, PBS injected or the *M. marinum* NCIMB 1298 group at 5 d.p.i. The group of fish infected with *M. marinum* NCIMB 1297 (lane 76 and 78) tested positive for mycobacterial DNA by the appearance of bands at 441 bp indicating the presence of the 65 kDa mycobacterial heat shock-protein.

Furthermore, successful infection of fish was confirmed in Chapter 3 by the presence of granulomas and bacteria stained with Ziehl-Neelsen within granulomas and the surrounding areas.

4.3.2.3 Validation of housekeeping gene expression

The gene expression of the three housekeeping gene candidates, Elong 1a, RPL13a and Beta actin, were compared using two different methods; (1) validation by geNorm and (2) Normfinder. Both methods apply an algorithm to calculate the stability of the reference genes.

Comparison by geNorm resulted in a stability value (M-value) of 1.6408 for Elong 1a and RPL13a whereas the value for Beta actin was higher (2.2910). By revealing a lower M value, both Elong 1a and RPL13a were suggested to be more stable than Beta actin for the infection model used and were thus more suitable as housekeeping genes.

The validation of the three housekeeping gene candidates with Normfinder resulted in a stability value of 0.2101 for Elong 1a identifying it as the most stable individual gene. Normfinder also calculated the best stability value for a combination of two genes with the result that a grouping of Elong1a with RPL13a would lead to a stability value of 0.1934.

Taking the results of the two validation methods into account, a combination of Elong1a and RPL13a was considered to be the most suitable choice and therefore these two housekeeping genes were used in the calculation of the gene expression changes of pro-inflammatory cytokines in this Chapter.

4.3.3 Gene expression of innate, pro-inflammatory cytokines interleukin 1 beta, tumor necrosis factor alpha and interleukin 12

Gene expression of the pro-inflammatory cytokines IL-1β, TNFα and IL-12 were determined following injection of two different isolates of *M. marinum* (NCIMB 1298 and NCIMB 1297). Two sets of control fish were used (no injection and PBS injection) to determine if infection *in vivo* by intra-peritoneal injection (i.p.), the traditional method for infecting zebrafish with *M. marinum* is suitable in such small individuals. It is possible that the process of injection could, in itself, cause such a strong cytokine response that it might mask the true immune response to the pathogen.

4.3.3.1 PBS injected fish

No significant differences were detected between non-injected fish and PBS-injected fish for any of the time points. Large standard errors, especially at 3 and 7 d.p.i for IL-1β and 1 d.p.i. for TNFα were, however, noted when the gene expressions of the PBS-injected fish were normalised to non-injected ones.

Comparison of the cytokine gene expression (mean ± SEM) across the sampling days, two of the three tested cytokines investigated revealed significant variation ($p<0.05$) between the different days. Although no significant differences were detectable in TNFα expression (Fig. 4-11B) over time, IL-1β expression decreased significantly at 3 d.p.i. (-0.98 ± 0.62) ($p=0.0277$) (Fig. 4-11A) and IL-12 displayed significant differences in the gene expression between 7 and 14 d.p.i ($p=0.0095$) (Fig. 4-11C). The expression decreased between these two sampling points from 1.82 ± 0.71 to -1.12 ± 0.85.

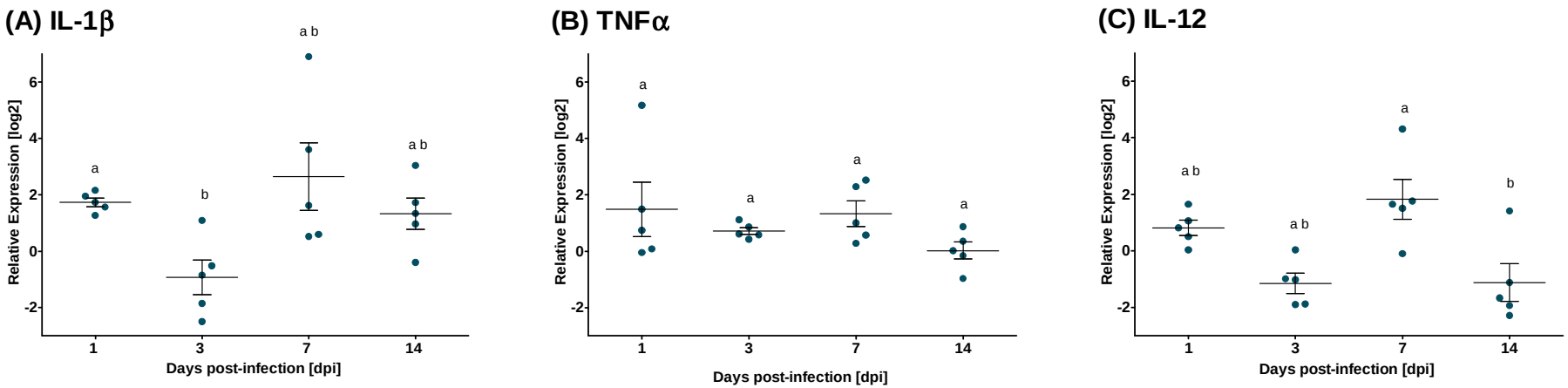

Figure 4-11: Kinetics of expression of pro-inflammatory genes (A) interleukin 1 beta, (B) tumor necrosis factor alpha and (C) interleukin 12 in the kidney of adult zebrafish experimentally injected with PBS. Data represent the relative expression (in log2 scale) as a vertical scatter plot of the individual fish (blue data points) including statistical mean value of individuals (horizontal black bar) and calculated standard error compared to non-injected control fish at the same time point and normalised to the housekeeping genes Elongation factor 1 alpha and Ribosomal protein L13a (n=5). Statistically significant differences ($p<0.05$) between sampling points were determined by a non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test and are indicated by different letters above data sets

4.3.3.2 Fish injected with *M. marinum* NCIMB 1298

The gene expression profiles for fish infected with the presumed non-infective *M. marinum* isolate NCIMB 1298 were calculated twice: (1) normalised to non-injected fish and (2) normalised to PBS-injected fish to determine whether the normalisation method has an impact on the gene expression on a secondary level as the direct comparison of non-injected and PBS injected fish revealed no significant differences.

The fish normalised to non-injected fish showed a significant up-regulation in the IL-1β expression at 14 d.p.i. (2.43 ±0.27, p=0.023) compared to the control fish In addition, and similar to the findings in the previous section, large standard errors of the calculated expression values were noticed (Fig. 4-12).The IL-1β and IL-12 expression profiles showed variations across sampling days (Fig. 4-12A and C) while the TNFα expression showed hardly any change at all (Fig. 4-12B). The variations in the gene expression profiles for IL-1β were significant between 3 and 14 d.p.i (-1.08 ± 0.43 and 2.43 ± 0.27, p=0.019). IL-12 gene expression was significantly down-regulated between 1 and 3 d.p.i (0.62 ± 0.48 to -1.82 ± 0.10, p=0.036).

Normalisation of fish injected with *M. marinum* NCIMB 1298 to PBS-injected fish displayed similar expression profiles for the three investigated cytokines but revealed changes regarding the significance of the results (Fig. 4-13). The IL-1β expression in *M. marinum* NCIMB 1298 injected fish was at no time significantly different to the PBS-injected ones (Fig.4-13A) which differs from normalisation to the non-injected ones. Variation across sampling time points was only significant for up-regulation of IL-1β expression between 7 and 14 dpi (-3.81 ± 1.58 to 1.10 ± 0.27, p=0.023). Similar to the results normalised to non-injected fish, TNFα expression showed no changes across sampling days when compared to PBS injected fish (Fig. 4-13B) and IL-12 expression showed also no significant variations (Fig. 4-13C).

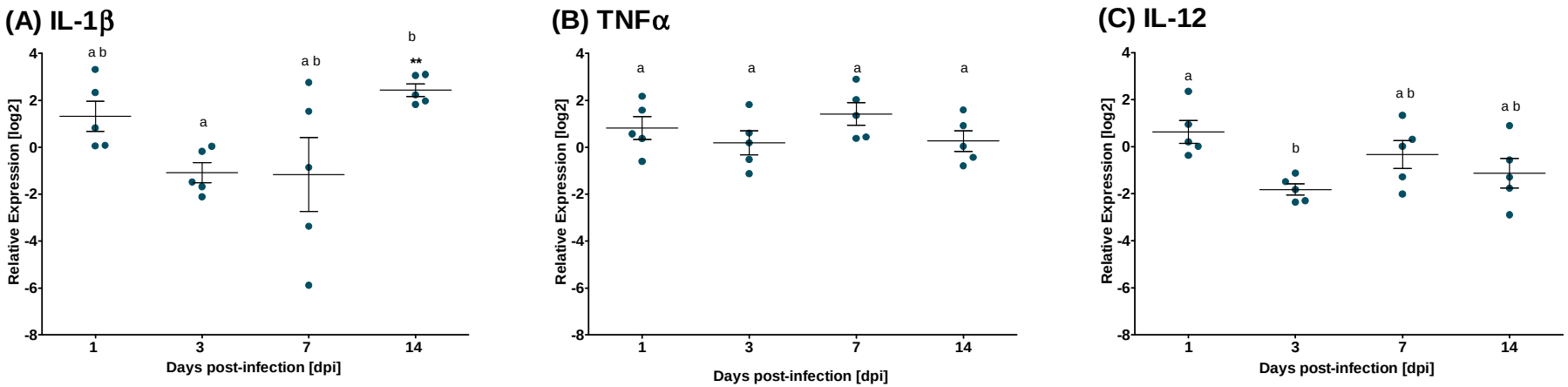

Figure 4-12: Kinetics of expression of pro-inflammatory genes (A) interleukin 1 beta, (B) tumor necrosis factor alpha and (C) interleukin 12 in the kidney of adult zebrafish experimentally infected with *M. marinum* NCIMB 1298 compared to non-injected fish. Data represent the relative expression (in log2 scale) as a vertical scatter plot of the individual fish (blue data points) including mean value for individuals (horizontal black bar) and standard error compared to non-injected control fish at the same time point and normalised to the housekeeping genes Elongation factor 1 alpha and Ribosomal protein L13a (n=5). Statistically significant differences (p<0.05) to the control group have been determined by a non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test and are indicated by a double asterisk (**). Statistically significant differences (p<0.05) between sampling points were determined by a non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test and are indicated by different letters above data sets.

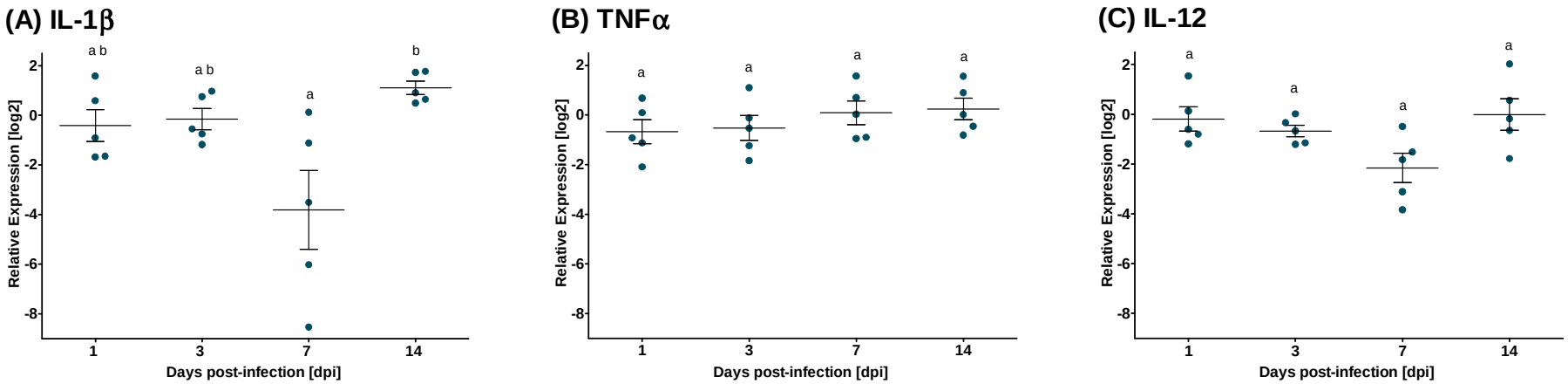

Figure 4-13: Kinetics of expression of pro-inflammatory genes (A) interleukin 1 beta, (B) tumor necrosis factor alpha and (C) interleukin 12 in the kidney of adult zebrafish experimentally infected with *M. marinum* NCIMB 1298 compared to PBS injected fish. Data represent the relative expression (in log2 scale) as a vertical scatter plot of the individual fish (blue data points) including mean value of individuals (horizontal black bar) and standard error compared to non-injected control fish at the same time point and normalised to the housekeeping genes Elongation factor 1 alpha and Ribosomal protein L13a (n=5). Statistically significant differences ($p<0.05$) between sampling points were determined by a non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test and are indicated by different letters above data sets.

4.3.3.3 Fish injected with infective *M. marinum* NCIMB 1297

The gene expression profile for fish infected with the *M. marinum* strain NCIMB 1297 (known to be pathogenic) were calculated and normalised using the same methods as described above in Section 4.3.4.2.

Similar to the results obtained from the fish infected with *M. marinum* 1298, the gene expression pattern of each of the investigated cytokines hardly changed when normalised to non-injected or PBS injected fish but significant differences did occur.

Normalisation to non-injected fish as controls showed significant up-regulation in IL-1β gene expression at 14 d.p.i. (2.52 ± 0.67, p=0.023) (Fig. 4-14A). No significant differences to the control group were detectable in TNFα and IL-12 expression (Fig. 4-14B+C). Comparison between the sampling points revealed no significant differences for any of the three analysed cytokines (Fig. 4-14A-C).

Changing the control group from non-injected to PBS-injected fish for normalisation, showed the same effect previously demonstrated in the *M. marinum* NCIMB 1298 injected fish. Significant differences in IL-1β expression when compared to the non- injected control group were no longer found when expression data was calculated relative to the PBS injected fish (Fig. 4-15A). Furthermore no significant differences in IL-1β and TNFα expression were detected across sampling days.

The IL-12 expression profile showed a significant up-regulation at 14 d.p.i. compared to the control group (3.41 ± 0.99, p=0.0384) (Fig. 4-15C). In addition, IL-12 showed differences between the sampling days, being significantly higher at 14 d.p.i. than at 1 d.p.i. and 7 d.p.i. (-0.29 ± 0.33 and -0.84 ± 0.51, p=0.0084).

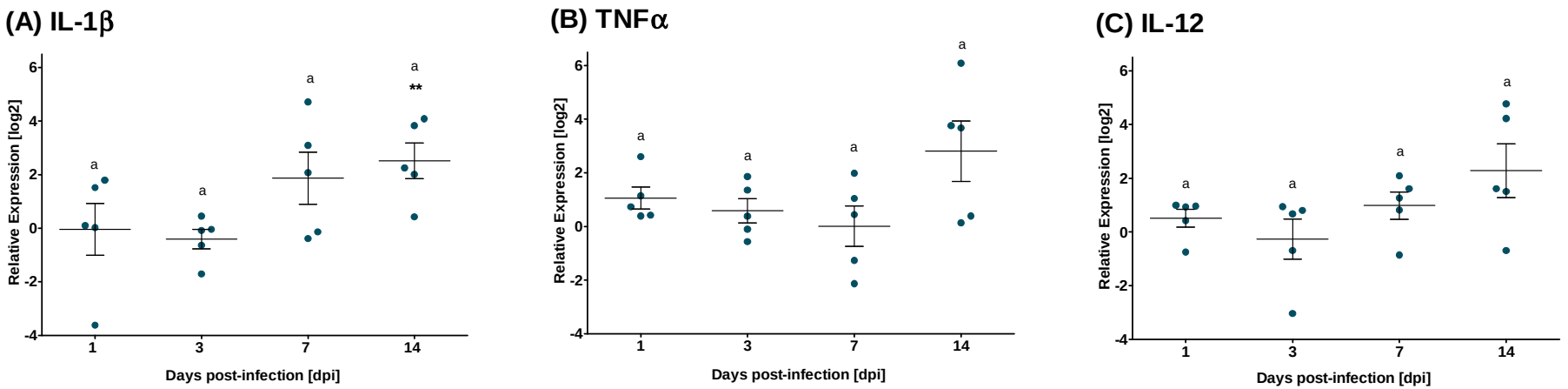

Figure 4-14: Kinetics of expression of pro-inflammatory genes (A) interleukin 1 beta, (B) tumor necrosis factor alpha and (C) interleukin 12 in the kidney of adult zebrafish experimentally infected with *M. marinum* NCIMB 1297 compared to non-injected fish. Data represent the relative expression (in log2 scale) as a vertical scatter plot of the individual fish (blue data points) including mean value of individuals (horizontal black bar) and standard error compared to non-injected control fish at the same time point and normalised to the housekeeping genes Elongation factor 1 alpha and Ribosomal protein L13a (n=5). Statistically significant differences ($p<0.05$) between sampling points were determined by a non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test and are indicated by different letters above data sets.

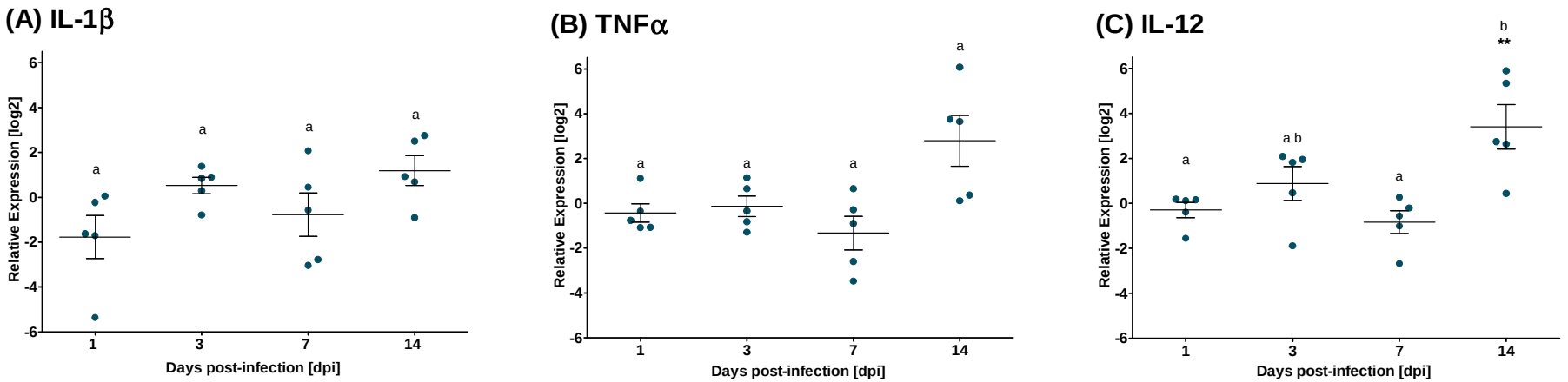

Figure 4-15: Kinetics of pro-inflammatory genes (A) interleukin 1 beta, (B) tumor necrosis factor alpha and (C) interleukin 12 in the kidney of adult zebrafish experimentally infected with *M. marinum* NCIMB 1297 compared to PBS-injected fish. Data represent the relative expression (in log2 scale) as a vertical scatter plot of the individual fish (blue data points) including mean value of individuals (horizontal black bar) and standard error compared to non-injected control fish at the same time point and normalised to the housekeeping genes Elongation factor 1 alpha and Ribosomal protein L13a (n=5). Statistical significant differences (p<0.05) to the control group have been determined by a non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test and are indicated by a double asterisk (**). Statistically significant differences (p<0.05) between sampling points were determined by a non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test and are indicated by different letters above data sets.

4.3.4 Expression profile of Interferon gamma in challenged fish

Interferon gamma (IFNγ) expression was only detected in zebrafish experimentally infected with *M. marinum* NCIMB 1298 and NCIMB 1297 after 14 d.p.i. (Fig. 4-16).

No significant differences could be detected between the different treatment groups when tested with Kruskal-Wallis and Dunn's multi-comparison *post hoc* test ($p=0.069$). Fish injected with *M. marinum* NCIMB 1297 exhibited a mean expression with a distinct larger standard error (3.291 ± 1.20) than the other three treatment groups (0.00 ± 0.2655 for non-injected fish, 0.711 ± 0.377 for PBS injected fish and 0.740 ± 0.456 for *M. marinum* NCIMB 1298 injected fish). Comparison of the individual responses in Figure 4-16 showed that the data points for the non-injected, PBS injected and *M. marinum* NCIMB 1298 injected fish were clustered more tightly than for the fish injected with *M. marinum* NCIMB 1297 which displayed a wider spread ranging from 0.32 to 7.04, explaining the large standard error.

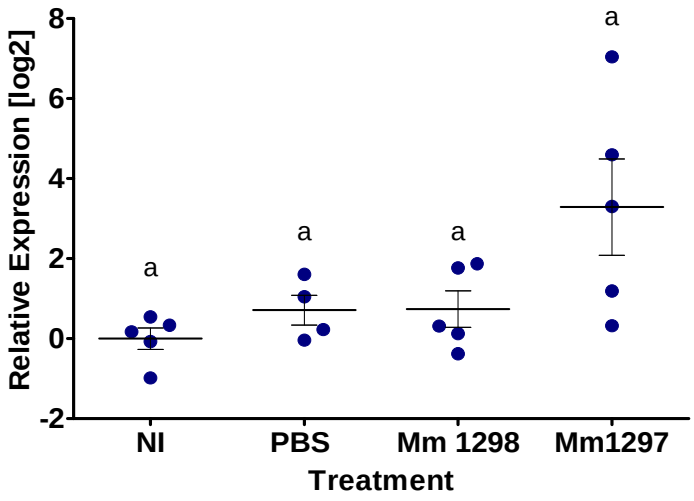

Figure 4-16: Relative Expression of interferon gamma in the kidney of adult zebrafish experimentally infected with *M. marinum* NCIMB 1297 (Mm1297), NCIMB 1298 (Mm1298) and control fish injected with PBS (PBS) or non-injected fish (NI) at 14 days post-infection. Data represent the relative expression (in log2 scale) as a vertical scatter plot of the individual fish (blue data points) including mean value of individuals (horizontal black bar) and standard error compared to non-injected control fish at the same time point and normalised to the housekeeping genes Elongation factor 1 alpha and Ribosomal protein L13a (n=5). Statistically significant differences ($p<0.05$) between sampling points were determined by a non-parametric Kruskal-Wallis test with Dunn's multi-comparison *post hoc* test and are indicated by different letters above data sets.

4.4 *DISCUSSION*

4.4.1 Development and optimisation of *in vitro* assays

4.4.1.1 Comparison of leukocyte isolation methods

The three most common density gradient based leukocyte isolation methods (34/51% Percoll, 37/54% Percoll and Histopaque) were used to isolate leukocytes from small brown trout prior the attempt to isolate these cells from much smaller zebrafish. No statistically significant differences between the three methods were observed. Histopaque was, however, selected as the method to use for further experiments as this was determined as the simplest and quickest method to use for a number of reasons. The density gradient was supplied as a ready to use solution. The desired volume was pre-aliquoted and if necessary could be warmed to the appropriate temperature before the cell suspension was subsequently layered on it and cells were separated by centrifugation. The band of mononuclear cells was clearly visible at the interface of the Histopaque and L-15 medium due to the different colours and hardly any contamination of red blood cells was observable. By comparison, Percoll was supplied as a stock concentration from which the desired gradient concentrations were diluted. The liquid chosen for the dilution of the Percoll appeared to have an effect on the visibility of the interphase between the two Percoll layers resulting in an easier differentiation of the interface when diluted with L-15 instead of HBSS or water.

Considering the reproducibility of leukocyte isolation with Histopaque and Percoll, the Histopaque isolation seemed to benefit from a number of advantages. The reproducibility appeared to be better standardised due to the ready-to-use gradient. The dilution of Percoll to the desired gradient concentration allowed a wider range of applications for this product but also increased the error due to the requirement to dilute the gradients appropriately. The gradients can be prepared in advance and therefore can guarantee the consistency of the gradients throughout an experiment

(Amersham Biosciences 2001) but across experiments the reproducibility might be affected. As mentioned, Percoll can be diluted to the desired concentration facilitating both continuous and discontinuous gradients for cell isolation (Amersham Biosciences 2001). Histopaque, on the other hand, is commercially available at a range of ready-to-use gradients including 1.077 g mL^{-1}, 1.083 g $mL^{-}1$ and 1.119 g mL^{-1}. By purchasing more than one of these Histopaque gradients, it is possible to layer one onto another and therefore isolate cells with a discontinuous gradient (Frei 2011).

Isolation of leukocytes by density gradient centrifugation is a common procedure for human and mammalian samples (Almeida *et al.* 2000; Munteanu and Dinu 2004; Chang *et al.* 2009) as well as for teleost samples (Braun-Nesje *et al.* 1981; Waterstrat *et al.* 1988; Secombes 1990; Crippen *et al.* 2001). The isolation of leukocytes from fish has been described in a variety of species including Atlantic salmon (Secombes 1990; Herath 2010; Kvamme *et al.* 2013), carp (Fujiki *et al.* 2003; Joerink *et al.* 2006; Ardó *et al.* 2010), rainbow trout (Frøystad *et al.* 1998; Stafford *et al.* 2001; MacKenzie *et al.* 2003); tilapia (Waterstrat *et al.* 1988) and goldfish (Wang *et al.* 1995; Neumann *et al.* 2000; Belosevic *et al.* 2006), using either Percoll or Histopaque. The results presented here showed that Histopaque is an acceptable alternative to Percoll for rapid isolation of total leukocytes. In mammalian and human research, this alternative method is already widely accepted and Histopaque or equivalent products are used in the majority of leukocyte isolation experiments. Confirmation of equivalency between Percoll and Histopaque has been shown by a number of authors (Almeida *et al.* 2000; McCoy Jr 2001; Munteanu and Dinu 2004; Chang *et al.* 2009). Percoll on the other hand is recommended for isolation of specific leukocyte populations.

4.4.1.2 Evaluation and characterisation of the adherent leukocytes

This Chapter aimed to establish not only a zebrafish kidney leukocyte culture but to further purify it to a primary monocyte / macrophage culture. To purify

monocytes / macrophages from leukocyte culture by adherence is an established quick and easy procedure widely accepted in mammalian and fish cell cultivation (Secombes 1990; Almeida *et al.* 2000; Wahl *et al.* 2006; McKinley 2007). Purification is performed by using the adherent properties of monocytes / macrophages. The isolated leukocyte culture is transferred into appropriate cell culture flasks or chambers and incubated for a number of hours to enable attachment of the monocytes / macrophages.

Despite the simplicity of this method, Almeida *et al.* (2000) pointed out some disadvantages of this purification method. They particularly focused on lymphocyte contamination of primary monocyte cultures originating from human peripheral blood which comprised up to 50% of the cultured cells within the first hour and still 25% after 24 h. The number of leukocytes isolated is dependent on a number of variables such as adherence time and number of washings, which makes it variable between researchers and also the blood donor might affect the lymphocyte number of itself (Almeida *et al.* 2000).

Using adherence, purification of the monocytes / macrophages from the other leukocytes was attempted from brown trout and zebrafish in the present study and contamination with small cells, possible lymphocytes, was noted. These cells exhibited a high nucleus to cytoplasm ratio and remained attached to the glass slide even after vigorous washing. Besides lymphocytes, these small leukocytes could also be progenitor macrophages. Progenitor or R1-type macrophages and their resemblance to lymphocytes have been described in primary goldfish kidney cell culture (Neumann *et al.* 2000; Barreda and Belosevic 2001; Belosevic *et al.* 2006).

Such R1-type or progenitor macrophages formed the majority of the cells besides non-macrophage cells in a primary goldfish culture on day 0 and had nearly disappeared by day 4 (Barreda and Belosevic 2001; Belosevic *et al.* 2006). These findings in goldfish agree with the findings in humans where lymphocytes represent up 25% or more of a primary peripheral blood mononuclear cell culture (Almeida *et al.*

2000). However, due to the small size of these cells and the high resemblance to lymphocytes, it was hard to distinguish whether small cells found in the current work were R1-type macrophages or lymphocytes. Both these types of cells are small and exhibit a high nucleus to cytoplasm ratio. Further differentiation needs to be performed by sorting the primary zebrafish kidney cell cultures with FACS or using specific staining techniques to determine whether the zebrafish kidney cell culture was a pure monocyte / macrophage culture; it was therefore referred to as zebrafish leukocyte culture instead.

4.4.1.3 Development of a zebrafish leukocyte mycobacteria infection assay

After successful establishment of leukocyte cell cultures from individual zebrafish kidneys, cultures were subjected to infection with *M. marinum* NCIMB 1297 and gene expression of infected and control cells was measured at 1, 3 and 6 h.p.i.

The results obtained suggested that the infection of the isolated kidney leukocytes was successful; however, these results have to be viewed with caution due to the low n number in cell counting. The number of cells counted in this study was lower in the infected wells than in the control wells at all time points. Presumably the cells in the infected wells had phagocytosed *M. marinum*, which then multiplied in the unactivated macrophages without restriction, leading to detachment of the cells which explained the resulting lower counts as presented in the results (Section 4.3.1.2.). This hypothesis is supported by Todar (2014) who indicated that uncontrolled growth of bacteria can lead to bursting of macrophages and a decrease in the cell number counted. It has also been shown that *M. tuberculosis* infecting murine or human macrophages induced atypical lysosomal cell death (Fratazzi *et al.* 1999; Lee *et al.* 2006; Hartman and Kornfeld 2011) and necrosis (Repasy *et al.* 2013). These macrophages displayed early features of apoptosis but instead of following the classical apoptotic pathway, these cells progressed quickly to necrosis, which did not

decrease the viability of *M. tuberculosis*. In this case apoptosis caused detachment of cells from the culture flask, resulting in lower cell counts.

No significant changes were detected in the extracted RNA yields during the course of the experiment. Although this was expected due to the low cell number, further optimisation of the RNA extraction methods in the future might result in larger and purer RNA yields. In addition, alternative quantification methods such as fluorescence-based RNA quantification (pers. comm. T. Herath, Institute of Aquaculture) might aid in quantifying the extracted RNA yields more precisely than the NanoDrop which shows inaccuracy in detection of values under 100 ng mL^{-1} (Bustin 2000; 2002; Dheda *et al.* 2004). Unfortunately, the low yield of RNA obtained from the cultured cells limited the number of immune genes that could be examined for the RT-qPCR and therefore the focus was set on two important innate immune response genes, IL-1β and TNFα, and the intracellular toll-like receptor TLR9.

Gene expression analysis of control and infected leukocyte cultures revealed a significant up-regulation of IL-1β and TLR9 at 1 h.p.i. compared to the control cells. At 3 h.p.i, only TLR9 remained significantly up-regulated. TNFα was at no time significantly up-regulated in comparison to the control cells. TLR9 plays an important role in the recognition of pathogens and subsequent activation of the innate immune response (Leifer *et al.* 2004). Up-regulation of the gene expression encoding this receptor confirmed the successful infection of the cultured leukocytes. This receptor is located in the intracellular compartment and is triggered by bacterial DNA and synthetic oligodeoxynucleotides (ODN), containing unmethylated CpG dinucleotides (Leifer *et al.* 2004).

IL-1β has been shown to be produced *in vitro* by murine and human macrophages infected *with M. tuberculosis* (Giacomini *et al.* 2001; Placido *et al.* 2006; Hartman and Kornfeld 2011). Expression of this cytokine was also shown to be up-regulated after *in vitro* infection of cultured macrophages (Giacomini *et al.* 2001; Hartman and Kornfeld 2011) as well as after efferocytosis of infected and apoptotic

neutrophils by macrophages (Placido *et al.* 2006). Hartman and Kornfeld (2011) identified IL-1β as an adherent signal which further induces expression of other cytokines in a direct or indirect way. IL-1β is known to participate in a variety of pathways and receptor interactions including recognition of PAMPs by macrophages (Vance *et al.* 2009; Fontana and Vance 2011). Therefore expression of IL-1β after recognition of the mycobacteria by the cultured leukocytes would have been expected in the experimental setup of this Chapter.

In contrast, the unchanged expression of TNFα in the infected wells in comparison to the control was unexpected. TNFα is known to play an important role in the immune response against pathogens such as *M. tuberculosis* (Flynn *et al.* 1995; Lin *et al.* 2007; Stockhammer *et al.* 2009; van der Vaart *et al.* 2012) and therefore an up-regulation in response to the infection would have been expected. The absence of TNFα expression during infection has been previously described in human and murine cell culture *in vitro* infection experiments (Balcewicz-Sablinska *et al.* 1999; Lee *et al.* 2006). Balcewicz-Sablinska *et al.* (1999) showed that TNFα was inactivated when human alveolar macrophages were infected with the virulent *M. tuberculosis* strain H37Rv. The macrophages released significant more of the anti-inflammatory cytokine IL-10 which supressed TNFα production. Infection with the attenuated strain H37Ra resulted in a normal TNFα response. Similar results have been found by Lee *et al.* (2006). Infection of bone marrow-derived murine macrophages with different doses of *M. tuberculosis* Erdman, *M. bovis* BCG Pasteur or rBCG-expressing GFP resulted in a different response of the macrophages depending on the infection dose. No differences in TNFα expression were detected in the response of the infected cells when they were infected with a high dose of any of the *Mycobacterium* strains. The authors suggested that this was explained by the fact that high dose infection triggered a TNF-independent apoptotic response, while the low dose response appeared to be TNFα dependent. The authors based this assertion on the observation that infection of macrophages with low doses of attenuated strains such as BCG or *M. tuberculosis*

H37Ra resulted in killing of the intracellular bacteria in TNFα mediated apoptosis, while infection with virulent strains such as *M. tuberculosis* H37Rv or *M. bovis* resulted in viable intracellular bacteria, which successfully suppressed apoptosis by the host cell (Lee *et al.* 2006).

The lack of TNFα response in this study might be explained by the same phenomenon. The experiment comprised an infection of zebrafish kidney leukocytes with a low dose of a virulent *M. marinum* stain. Therefore the conditions in this study were similar to those in the experiment performed by Lee *et al.* (2006). It would be interesting to investigate further cytokine expression such as IL-10 in the future to confirm the suppression of apoptosis. In addition, infection with a non-infective *M. marinum* strain such as NCIMB 1298 and the application of various infection doses might be helpful for further understanding the immune response to mycobacterium-infections.

In conclusion, the suppression of the apoptotic response by a virulent *Mycobacterium spp* strain *in vitro* corresponds to the situation observed *in vivo,* where it is generally accepted that virulent *M. tuberculosis* suppresses the immune response of the human host from first contact (Clatworthy *et al.* 2009; Urdahl *et al.* 2011). Therefore it is not surprising that virulent *M. marinum* NCIMB 1297 might display a similar suppression mechanism in zebrafish.

4.4.1.4 Concluding remarks for the *in vitro* assay

The preparation of *in vitro* cell cultures from zebrafish is still not as advanced as for other model organisms (Collodi *et al.* 1992; He *et al.* 2006). Only five permanent zebrafish cultures have been listed in ATCC so far comprising 2 fibroblast cultures derived from zebrafish embryos, 1 epithelial cell culture derived from the liver of adult zebrafish and 2 fibroblast cultures derived from the caudal fin of adult zebrafish (ATCC, 2012). The number of non-listed permanent cultures is hard to estimate. Listing all existing and new developed cell cultures in an international cell repository

would facilitate an easier way to exchange and purchase cultures (Pandey 2013). Very few studies have utilised primary adult zebrafish kidney cultures (Novoa *et al.* 2006; Palić *et al.* 2007; Rodriguez *et al.* 2008; Hohn *et al.* 2009). The existing published procedures comprise the removal of the kidneys and homogenization through a cell strainer into culture medium. The resulting cell suspension is transferred into appropriate cell culture flasks before further use (Novoa *et al.* 2006; Rodriguez *et al.* 2008). In addition, a large number of kidneys, 45-140 were pooled in these publications to achieve a high number of isolated cells. In our study, cells from individual kidneys were isolated with subsequent purification by attachment overnight. The dead and non-attached cells were removed by washing the cells with culture medium. These points together resulted in smaller numbers of cells to work with than in other published studies. In this study, it was shown that it is possible to isolate zebrafish leukocytes from individual fish as potential macrophage primary cultures, however, further optimisation has to be carried out to determine the purity of the cultures and perform further *in vitro* infection studies to confirm the results obtained. Nevertheless, such *in vitro* assays show potential for future research.

In vitro cultures are useful tools to investigate the response of particular immune cells, such as macrophages used in the present study, and to pathogens without the interaction of this particular cell with other immune cells. It is a rapid and inexpensive method for achieving first insights into the immune response of a particular cell and studies in human and mice have shown that *in vitro* studies in general are able to predict *in vivo* activity (Sonneveld *et al.* 2006).

However, especially in regard to the macrophage as a key in human tuberculosis infections, the interaction of this immune cell with other participants of the immune response such as T cells, is important for the proper functioning and the activation of the macrophage and subsequently the killing of the pathogen. Therefore, the results of *in vitro* assays have to be confirmed by *in vivo* studies in which the entire immune response at a certain time point is available. These comparative studies would

also aid in detecting defects or delays in the immune response towards a certain pathogen, when results of *in vitro* and *in vivo* studies lack correlation.

4.4.2 Infection of zebrafish with *M. marinum in vivo*

Infection studies were also performed *in vivo* and host responses investigated. This involved optimising RNA extraction methods for small tissue samples, determining the best housekeeping genes to use during *Mycobacterium* infection and establishing whether injecting such small fish can of itself influence gene expression.

4.4.2.1 Optimisation of RNA extraction with TriReagent

TriReagent was used to extract RNA from fresh individual zebrafish kidney tissue samples. This method is one of the most popular extraction methods and is convenient for economical, quick, efficient isolation of total RNA, especially in studies with large sample sizes or for those with a limited budget. Due to the low amount of tissue available in this study as a result of fish size, the standardised method was modified (Pérez-Osorio and Franklin 2008) and an RNA carrier, LPA was added to the extraction method. Other adjustments were also introduced, *i.e*. over-night precipitation at -20°C and longer centrifugation steps (personal communication E. McStay and J. Heumann, Institute of Aquaculture, Stirling, UK) to increase the resulting RNA yields. The final optimised protocol gave better and more consistent yields of RNA than the original method. Using TriReagent, the final RNA pellet can be difficult to re-suspend (Rio *et al.* 2010), especially if the air-drying step was performed longer than indicated by the manufacturer's protocol. This extended drying was necessary as all the ethanol would not be removed from the pellet prior to air-drying as the RNA pellet was so small and fragile. Pellets were hardly visible after the ethanol washing steps and to avoid losing the pellet by removing all ethanol with a pipette, the final amount of the ethanol was left in the tube with the RNA pellet, which prolonged the air drying step and might have led to an over-dried pellet in a large number of

samples. To facilitate re-suspension of the RNA in dH2O, the samples were incubated overnight at 4°C, according to Wu *et al.* (2011) who showed that storage of RNA in dH2O at 4°C is possible for up to 7 days without having a negative effect on the stability of the RNA. The resulting RNA was within an acceptable yield range for continuing with cDNA synthesis and subsequent qPCR analysis after quantifying the concentration using the NanoDrop. A quality check of the RNA by running some of the RNA on an agarose gel, as advised by the manufacturer's protocol was not possible due to the low amount of RNA obtained. A minimum amount of 200 ng is needed to visualise RNA on an agarose gel with ethidium bromide (Invitrogen 2014) and with a maximum of 1000 ng obtained following RNA extraction, insufficient RNA was available for a quality check because the RNA was required for cDNA synthesis and gene expression analysis.

4.4.2.2 Basis for choosing relative methods over alternative methods for normalisation of data

Another important aspect of comparable gene expression analysis is the right choice of normalisation method. The expression values of the tested samples can either be normalised to the total amount of RNA (absolute quantification) or to the expression of marker genes, so-called "housekeeping" genes (relative method) (Bergkvist *et al.* 2008). The absolute method is based on a standard curve and therefore the determination of the quantity and the assurance of the quality of the RNA used for preparing the standard curve have to be as good as possible (Life Technologies Corporation 2012) The spectrometric method on which NanoDrop is based to determine RNA quantities is unreliable for concentrations lower than 100 ng μl^{-1} and other factors such as contaminants and / or residual DNA despite DNAase treatment may interfere with the accuracy of the NanoDrop results (Bustin 2000; 2002; Dheda *et al.* 2004). Furthermore, the relative method is widely used and is the most favoured one to investigate changes in gene expression analysis and was hence used

in this study. The basis of the relative method is the comparison of the ct value of a housekeeping gene to that of a gene of interest. As long as the same sample dilution is used to measure the expression of the housekeeping gene and the gene of interest throughout the whole experiment, the actual amount of RNA can be ignored.

4.4.2.3 Validation of housekeeping genes

Fundamental to this gene expression analysis is the validation of the most appropriate housekeeping genes for the normalisation. The ideal housekeeping gene has to be stably expressed in the tissue of interest under the investigated conditions (Andersen *et al.* 2004; Bergkvist *et al.* 2008). In this study, Beta actin, Elong 1a and RPL13a were tested as potential housekeeping genes. Beta actin was chosen because it is one of the most frequently used housekeeping genes and is commonly used in gene expression analysis in adult zebrafish infection models (Alonso *et al.* 2004; Pressley *et al.* 2005; Rodriguez *et al.* 2008; Vojtech *et al.* 2009); while Elong 1a and RPL13a were selected due to their recommendation in papers focusing on the validation of housekeeping genes in zebrafish (Tang *et al.* 2007; McCurley and Callard 2008; Lin *et al.* 2009b).

The Normfinder and geNorm results in the current study showed that Elong 1a and RPL13a were more stable than Beta actin and a combination of RPL13a and Elong 1a is the most stable solution.

Unfortunately, no information was available on housekeeping gene validation in zebrafish during disease challenges and therefore the practicability of Elong 1a as a housekeeping gene could only be determined and discussed in regard to the findings in other fish. The same applies for RPL13a and Elong 1a.

Elong 1a is one of the most frequently used housekeeping genes in expression profiling and has been validated as a suitable control in a variety of fish including zebrafish (Infante *et al.* 2008; McCurley and Callard 2008; Lin *et al.* 2009b; Øvergård *et al.* 2010; Aursnes *et al.* 2011; Su *et al.* 2011). The majority of housekeeping gene

validation studies in fish have focused on the stability of a potential reference gene over different stages of development or between different tissue samples. In combination with a stressor, either pathogenic or environmental, validation of Elong 1a has provided contradictory results with studies supporting (Jorgensen *et al.* 2006; Ye *et al.* 2010; Aursnes *et al.* 2011) or rejecting the use (Dang and Sun 2011; Su *et al.* 2011) of this gene.

RPL13a, in particular, has only been validated as a suitable housekeeping gene in comparison of different zebrafish tissues (Tang *et al.* 2007) without the influence of a pathogenic stressor. However, other members of the ribosomal protein family have been used in mammalian studies and have been successfully tested as suitable reference genes in fish (Jorgensen *et al.* 2006; Zhang and Hu 2007; Øvergård *et al.* 2010; Dang and Sun 2011). These results confirm that ribosomal proteins might be a potential source for new housekeeping genes for fish during disease challenge, but further validation has to be performed especially when transferring the gene between species or applying new experimental setups.

The finding that Beta actin was not applicable in this study as a housekeeping gene is supported by a number of publications focusing on a variety of vertebrate studies, ranging from development to disease research (Piehler *et al.* 2010; Ye *et al.* 2010). Additional publications focus on the combination of *M. tuberculosis* infections with other pathogenic mycobacteria (Dheda *et al.* 2004; Taylor *et al.* 2007). Dheda *et al.* (2004) showed variation of standard housekeeping genes, *i.e.* Beta actin, Elong 1a and GAPDH in human blood and PBMC cultures challenged with *M. tuberculosis* and discovered that none of the normally used housekeeping genes except HuPO was applicable to the study. Besides the numbers of papers supporting the fact that Beta actin and Elong 1a are unsuitable as housekeeping genes, a wide number of researchers found that Beta actin is stable (Peña *et al.* 2010), therefore giving a basis for further use.

The results of this study did not offer a solution to the complex problem of using Beta actin and Elong 1a as housekeeping genes. The stability values calculated using Normfinder were not ideal and showed variability within all the three housekeeping genes between the different sampling days in the kidney of mycobacteria infected zebrafish. The results obtained support existing guidelines recommended by other researchers (Vandesompele *et al.* 2002; VanGuilder *et al.* 2008): (i) if economically possible, test potential housekeeping genes prior to the experiment under similar conditions by using commercially available kits like geNorm (PrimerDesign). These housekeeping validation kits might be a quick and effective way to find the appropriate genes despite their elevated costs. However, it was not possible to use these kits in the current study and therefore the choice of housekeeping genes was guided by work already published using zebrafish; (ii) it is advised to use more than one housekeeping gene, especially when housekeeping validation kits are not applicable, they should be validated with freely available programs such as Normfinder or Bestkeeper before using them in the actual experiment.

Normalisation to a single reference gene is not considered good practice but is still applied in the majority of publications focusing on zebrafish immune response to infectious agents (Pressley *et al.* 2005; Rojo *et al.* 2007) and furthermore most of the time, the chosen housekeeping genes, i.e. Beta actin or Elong 1a, are not as stable as expected. Regarding the general problem with validation of the appropriate housekeeping genes, choosing more than three genes would have been desirable. Due to the limitation of sample RNA this was not possible.

Recently, new potential housekeeping genes have been used in some zebrafish infection studies. These genes have been chosen and validated by microarray (Bahr *et al.* 2009; Noriega *et al.* 2010), which appears to be a methodology for a successful choice of candidates. In humans and other mammalian models, this approach to selection is quite common but requires a microarray analysis based on the same conditions as the future experiment for which the reference gene will be used or

a large number of microarrays of the investigated species to choose possible housekeeping gene candidates, which are stably expressed under various conditions.

4.4.2.4 Confirmation of successful infection with *M. marinum*

At 5 d.p.i. DNA was extracted from the challenged fish as well as from the non-injected control fish and tested for the presence of mycobacteria by PCR. Only two of the 5 fish infected with NCIMB 1297 were tested positive for mycobacteria. As seen in Chapter 3, the histological detection of mycobacteria was not possible until 11 d.p.i. confirming that early detection of injected *M. marinum* is difficult. Furthermore, the organs where mycobacteria are normally first detected, such as kidney or peritoneal cavity, were not used for DNA extraction as these had been used for host response analysis and therefore this complicated the picture of detection. Despite this, the visible bands on the agarose gel following PCR, in combination with the results in Chapter 3, confirmed a successful infection of the challenged zebrafish with *M. marinum* NCIMB 1297, whereas the other treatments, *M. marinum* NCIMB 1298 and PBS, appeared to cause no infection.

4.4.2.5 Discussion of the sample size

The number of fish sampled per treatment at each time point was n = 5, which was relatively low and therefore left results highly susceptible to individual variation, explaining the large standard deviations in the study. The sample size of n = 5 per treatment resulting in a total n of 20 per sampling point was chosen according to how many fish it was possible to sample per day with subsequent homogenisation of the sampled tissue in TriReagent. The decision to include the homogenisation of the kidney tissue on the sampling day and not store it in a RNA preserving liquid such as RNAlater was made to minimize the loss of important kidney by transferring the small and fragile tissue from RNAlater into TriReagent or by accidently removing tissue by removing the RNAlater from the stored tissue.

This small sample size gives a low power and therefore minimises the possibility of demonstrating significant differences (Button *et al.* 2013).

4.4.2.6 Comparison of the pro-inflammatory immune response of the two control groups, non-injected and PBS-injected fish

The experimental design included two control groups, non-injected fish and PBS-injected fish. The significant variation across sampling days could be explained by the fact that for each sampling day different individual fish were utilised because repeated sampling was not possible. It was concluded that the injection itself had no effect on the gene expression of the genes investigated in the kidney. However, it still has to be determined whether other immune related organs such as the spleen or the immune responses in the coelomic cavity, the location of entry, are affected by the injection procedure. Administration of the appropriate volume as well as the choice of anaesthesia and the right injection equipment are essential to limit harmful side effects during and after the procedure (Morton 2000; Kinkel *et al.* 2010). The injected volume should be as small as possible. Other authors indicate that an injection volume of 10 mL per kg is recommended (Morton 2000), resulting in an infection volume of 10 µL for a 1 g zebrafish. The zebrafish used in this thesis had a weight between 0.4 and 1.2 g, thus an appropriate injection volume around 4-12 µL per fish. The volume administered in this study was actually 20 µL per fish, injected with a 30 gauge needle. As fish were so small this procedure does result in damage to the abdominal wall and therefore alternative challenge methods should be investigated for the future *e.g.* immersion or co-habitation. Intraperitoneal injection is one of the main methods used to experimentally infect zebrafish and was originally adapted from larger fish (Kinkel *et al.* 2010). The method allows the administration of an exact dose of an infectious agent where other methods like oral administration or immersion are not as reliable (Morton 2000; Kinkel *et al.* 2010). Injection protocols chosen in this study reflect those

previously published by other researchers (Neely *et al.* 2002; Lin *et al.* 2007; Phelps *et al.* 2009) and therefore the injected dose was considered appropriate.

In aquaculture, administration of vaccines to small fish by immersion is recommended rather than by injection, in order to limit the costs as well as the side effects (Sommerset *et al.* 2005). Immersion would be an alternative method for infecting zebrafish and is frequently used in combination with drugs or other bacterial pathogens (Pressley *et al.* 2005; Watzke *et al.* 2007; Stewart *et al.* 2011). The problems with this method, especially with *M. marinum*, are that (i) the number of bacteria taken up by individual fish cannot be established (Kinkel *et al.* 2010), resulting in a presumably wide deviation of cfu per fish and (ii) immersion studies with *M. marinum* in a shared aquaculture facility requires high bio-security measures to avoid accidental infection in other experiments through contamination by water. Furthermore, it has been shown that immersion challenges with successful infection of zebrafish requires pre-treatment of fish in the form of scale abrasion or similar injuries (Neely *et al.* 2002; Pressley *et al.* 2005; Lin *et al.* 2007; Moyer and Hunnicutt 2007). In experimental immersion challenge, Pressley and her colleagues proved that injury of adult zebrafish increased their susceptibility to *Edwardsiella tarda* infection, whereas non-injured fish displayed no susceptibility at all. Similar results were found by Neely *et al.* (2002) who infected zebrafish with *Streptococcus* and other studies undertaken in other fish species (Darwish 2007). Pre-treatment of challenged fish by scale abrasion or other injuries was not permitted on the animal project licence used in this study. Perhaps alternative less invasive methods, such as chemical treatment, would enable infection by the immersion route. For example, use of hydrogen peroxide has been shown to enable infection of rainbow trout with *Flavobacterium psychrophilum* (Henriksen *et al.* 2013).

4.4.2.7 Immune response to *M. marinum* 1298

The significance of the gene expression results with regard to pro-inflammatory cytokines in the kidney of adult zebrafish infected with *M. marinum* NCIMB 1298 depended on whether the gene expression had been calculated relative to non-injected fish or to PBS-injected fish. No significant differences were detected when the gene expression was calculated relative to the PBS injected fish and, except for IL-1β expression at 14 d.p.i., no changes were detected in relation to the non-injected fish.

One strain of *M. marinum* (NCIMB 1298) is considered infective only in *Neon tetras* (NCIMB Ltd 2013). This study supports this observation given that no differences were detected with respect to the two control groups and it is concluded that the fish were probably able to eradicate the bacteria as expected in the case of a non-infective bacterium. Gene expression of pro-inflammatory cytokines was measured in the kidney of fish injected into their peritoneal cavity. Therefore, a response in the kidney would only have been expected if the local innate response was not able to eradicate the bacteria, allowing the pathogens to spread through the host, inducing IL-12 production from infected macrophages to activate the adaptive immune response (Abbas and Lichtman 2003). If NCIMB 1298 was non-infective to zebrafish, the local immune response should have been able to control and eradicate the infection and therefore an infiltration of the bacteria into the kidney was not expected nor the activation of the induction of the Th1 adaptive response via IL-12 production. Eradication of a pathogenic stimulus should have occurred within 7 days as has been published in mice (Abbas and Lichtman 2003).

The significant up-regulation of IL-1β at 14 d.p.i. in the kidney of NCIMB 1298 infected fish when non-injected fish were used as the control group seems to be a combined effect caused by the injection and the bacteria. This conclusion is based on findings that the significant difference was only present with respect to the non-injected fish and not to the PBS-injected ones. If the effect was caused solely by the injection,

all three injected fish groups should have been significantly different from the non-injected ones and if the injected bacteria were the trigger, both bacteria-injected strains should have been significantly different to the non-injected and PBS injected fish. In humans, at 14 d.p.i. the tissue remodelling in wound healing is initiated alongside angiogenesis and re-epithelialisation (O'Loughlin and O'Brien 2011), whereas in the zebrafish model of wound healing, healing is nearly completed after 28 days post-wounding and the wounded area is not distinguishable from the unwounded tissue anymore (Richardson *et al.* 2013). Old, dead or destroyed cells will be replaced and transported to the kidney, which is responsible for the clearance of substances and blood filtering in humans as well as in fish (Christiansen *et al.* 1996; Agbede *et al.* 2012). It is thus possible that some extracellular products or remains of the injected bacteria, which have been controlled by the local immune response at the injection site were transported to the kidney and triggered a response in the form of IL-1β expression due to recognition of PAMPs by local macrophages. It has been previously reported that these PAMPs are present on all bacteria, pathogenic and non-pathogenic and can therefore can trigger an immune response (Fontana and Vance 2011). Vance *et al.* (2009) have further indicated that besides PAMPs, the host response possesses the ability to recognise other structures on the bacterial surface which aid in distinguishing dead from live bacteria and pathogenic from non-pathogenic. Triggering of an IL-1β response by a non-pathogenic bacterium has been previously described (Sander *et al.* 2011). However, Sander *et al.* (2011) proposed that only viable bacteria can trigger an IL-1β response due to their ability to induce cleavage of pro-IL-1β. Their findings were based on *in vitro* experiments on murine bone-marrow-derived macrophages and therefore raise the question of how comparable this is with *in vivo* experiments.

M. marinum NCIMB 1298 is known to produce extracellular products (Chen *et al.* 1996; 1997; 1998; NCIMB Ltd 2013) and it has been previously shown that injection of *M. marinum* NCIMB 1298 extracellular products into the swim bladder of Nile tilapia

triggers a non-specific immune response in the form of an elevated serum lysosome activity for up to 8 days (Chen *et al.* 1998). The same authors also reported that i.p. injection of *M. marinum* NCIMB 1298 extracellular products in rainbow trout elevated the non-specific and specific immune response for up to 10 weeks with peaks in the phagocytic activity detected at 2, 6 and 10 weeks post-injection (Chen *et al.* 1996). These findings support the hypothesis that the significant up-regulation of IL-1β in the kidney of i.p. injected zebrafish might be partially induced by the extracellular products produced by *M. marinum* NCIMB 1298. It has to be further investigated whether the existence of similar peaks of immune response, to those described in the phagocytic activity of rainbow trout (Chen *et al.* 1996), can also be found in zebrafish IL-1β expression when monitored over a longer time period.

4.4.2.8 Immune response to *M. marinum* 1297

Zebrafish infected with the pathogenic *M. marinum* strain NCIMB 1297 displayed the same significant up-regulation of IL-1β at 14 d.p.i. as described for *M. marinum* NCIMB 1298 injected fish in the previous section. Furthermore, IL-12 gene expression was also significantly up-regulated at 14 d.p.i. compared to PBS-injected fish. These findings support the hypothesis that up-regulation of IL-1β at 14 d.p.i. results from the combined effect of injection / wound healing and injection of bacteria but further leads to the conclusion that in this case, the bacteria which trigger the immune response in the kidney are still viable because of the significant IL-12 response. This is supported by the findings in Chapter 3 where *M. marinum* NCIMB 1297 was visualised by Ziehl-Neelsen staining in the kidney of infected zebrafish by 14 d.p.i.

The innate pro-inflammatory response to intracellular bacteria, as described in textbooks, results in an up-regulation of IL-1β, TNFα and IL-12 within the first 7 days (Abbas and Lichtman 2003; Divangahi *et al.* 2010; Shaler *et al.* 2012). These cytokines are important at initial stages of infection in triggering the cytokine cascade, which

recruits and activates macrophages and stimulates the adaptive immune response (Pressley *et al.* 2005). The absence of an early pro-inflammatory response at the site of initial infection is an important mechanism by which *Mycobacterium spp* delays the triggering of the adaptive immune response (Shaler *et al.* 2012) and has been demonstrated in human and mice infected with *M. tuberculosis* (Divangahi *et al.* 2010; Shaler *et al.* 2012) as well as in zebrafish infected with *M. marinum* (Parrish *et al.* 1998; van der Vaart *et al.* 2012). Adult zebrafish infected with a low dose of NCIMB 1297 or *M. marinum* Mma20 showed no pro-inflammatory response at 1 d.p.i. (Parikka *et al.* 2012; van der Vaart *et al.* 2012). Only when a high dose of *M. marinum* NCIMB 1297 was injected, (Parikka *et al.* 2012) or the infected zebrafish displayed signs of the disease as in the case of Mma20 injected fish at 6 d.p.i. (van der Vaart *et al.* 2012), was a response in pro-inflammatory gene expression detectable. By delaying the innate pro-inflammatory immune response, it has been demonstrated that the differentiation towards Th1 cells is delayed as well and subsequently the response of the adaptive immune system (Shaler *et al.* 2012). In mammals, IL-12 is believed to be one of the major inducers of Th1 differentiation (Fig. 4-17) (Sneller 2002; van Crevel *et al.* 2002; Hamza *et al.* 2010) and by inhibiting or delaying the release of this cytokine, the induction of adaptive immunity will be delayed or inhibited as well.

It has been shown that mice infected with *M. tuberculosis* display an absent early innate inflammation response in their lungs which delays the recruitment of innate immune cells to the lung (Shaler *et al.* 2012). The authors further mentioned that the reason for the absence of the early response was connected to the mannose-capped lipoarabinomannan (ManLAM) in the mycobacterial cell wall, which binds to the mannose receptor (MR) of the responding macrophage and triggers the anti-inflammatory response. Induction of this inflammation response impaired the pro-inflammatory response and therefore delayed the immune response in infected mice up to 5 days post-infection (Shaler *et al.* 2012). ManLAM is a component of all slow-growing, pathogenic mycobacteria strains (Rajni and Meena 2011; Welin 2011) and

therefore it is possible that *M. marinum*, like *M. tuberculosis*, binds to the MR and induces the anti-inflammatory pathway and therefore delays the recruitment of innate immune cells. A delay of the innate immune cells could explain the lack of changes in gene expression of IL-1β, TNFα and IL-12 compared to PBS injected fish over the first 14 days. Regarding the results of this study, it would be interesting to know how anti-inflammatory cytokines such as TGFβ and IL-10 were expressed over the first 14 days post-infection. Unfortunately, due to the limited amount of RNA which was available, only a restricted number of cytokines were able to be tested in the present study.

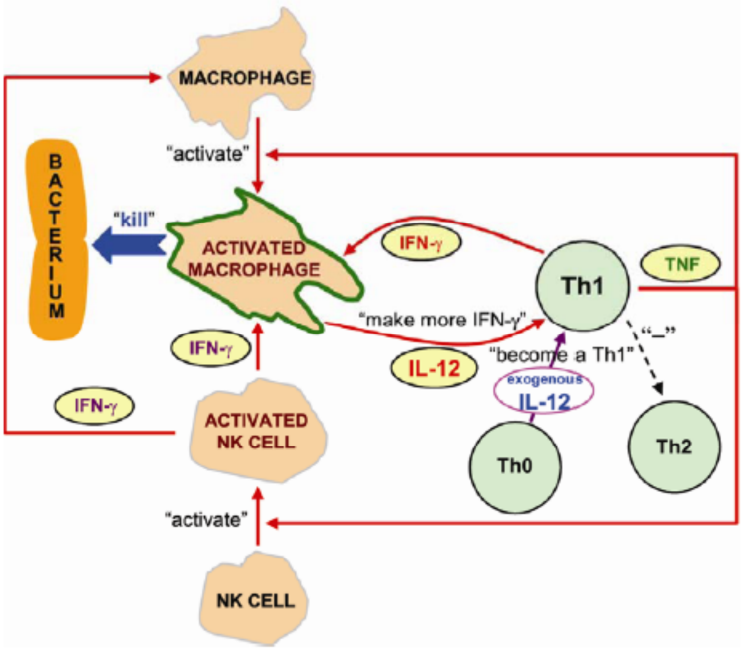

Figure 4-17: Local IL-12 therapy stimulates Th cells to secrete Th1 cytokines. Exogenous IL-12 application creates an environment rich in IL-12 around the infection site. In such a local environment, newly activated Th cells, responding to the presence of bacteria, exit the blood and are influenced to become Th1 cells and secrete more Th1 cytokines and activate macrophages and NK cells. Activated macrophages will produce more IL-12 and via positive feedback, cell-mediated immunity can be promoted to battle bacteria thereby leading to the prevention of infection. Taken from Hamza *et al.* (2010)

An important feature of the Th1 cells is the release of IFNγ to activate the infected macrophages to increase their ability to kill the ingested bacteria (Kaufmann 2002; Sneller 2002; Shaler *et al.* 2012; Silva Miranda *et al.* 2012). It has been demonstrated that in humans infected with *M. tuberculosis,* the adaptive immunity requires 5-6 weeks to develop (Divangahi *et al.* 2010), although T cell arrival at the site

of infection was detectable between 18 and 20 d.p.i. (Shaler *et al.* 2012). In mice the first T cells were able to be detected at 8-11 d.p.i. (Fig. 4-18) (Divangahi *et al.* 2010; Urdahl *et al.* 2011).

In this study, no significant changes were detectable in the IFNγ expression over the first 14 days of infection, although individual responses displayed a wide range of variation, implying that some fish might already have induced an IFNγ response while some had not. Similar to the IL-12 response, a positive regulation of IFNγ would have been expected earlier following exposure to the intracellular pathogen (Abbas and Lichtman 2003) and therefore agreeing with the hypothesis of a delayed immune response due to *M. marinum* infection.

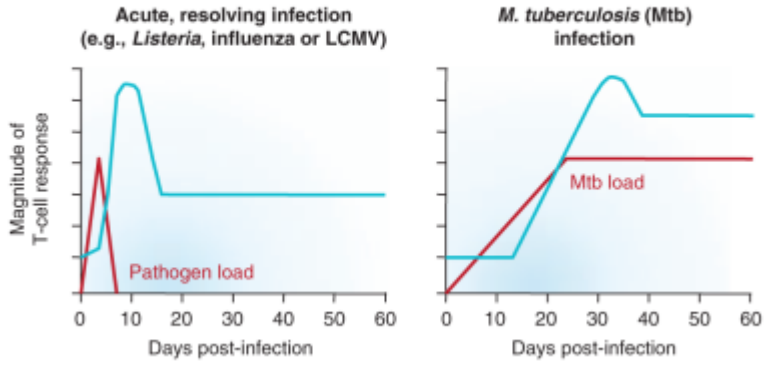

Figure 4-18: Delayed onset of adaptive immunity in mice compared with other infections. In acute, resolving infections, T-cell responses are initiated 3–5 days after initial infection; they peak 7–8 days after infection, and subsequently contract to establish memory populations. After aerosol infection with M. tuberculosis, T-cell responses are not initiated until 9–11 days after infection, peak several weeks after infection, and bacteria are not eliminated. LCMV, lymphocytic choriomeningitis virus. Taken from Urdahl *et al.* (2011)

Manipulation of the host to delay the immune response is known to occur in a variety of intracellular pathogen infections such as those caused by *Francisella spp.*, *Listeria monocytogenes, Salmonella typhimurium, Streptococcus spp.* and *Mycobacterium spp.* (Barker *et al.* 1998; El-Etr *et al.* 2001; Stamm *et al.* 2003; Vojtech *et al.* 2009; Benard *et al.* 2011; Welin 2011), but only in the case of *Mycobacterium spp.* is the delay in the mammalian immune response significant in comparison to that

of other intracellular pathogens (Divangahi *et al.* 2010; Shaler *et al.* 2012). In fish experimentally infected with viruses or intracellular pathogens, IFNγ expression was normally detectable within 7 d.p.i. (McBeath *et al.* 2007; Vojtech *et al.* 2009). Atlantic salmon infected with infectious pancreatic necrosis virus (IPNV) and infectious salmon anaemia virus (ISAV) displayed a significant up-regulation of IFNγ at 6-7 d.p.i. with return to a normal level at 8 d.p.i. (McBeath *et al.* 2007). Similar results have been shown in adult zebrafish infected with *Francisella noatunensis*, where IFNγ was significantly up-regulated at 3 and 7 d.p.i. (Vojtech *et al.* 2009). However, injection of the same bacterial species into Atlantic cod resulted in a delayed response of IFNγ and an up-regulation of cytokine did not occur until 15 d.p.i. (Ellingsen *et al.* 2011).

The current *in vivo* study showed an up-regulation of IL-12 but no changes in TNFα or IFNγ expression in the first 14 days. However, results from another experiment (not reported here) showed a significant up-regulation of TNFα at 28 d.p.i ($p=0.04$) and agreed with the findings of Ellingsen *et al.* (2011). This late response to *M. marinum* isolate NCIMB 1297 might indicate a delayed innate immune response as well. The findings in this study are further supported by the results of Parikka *et al.* (2012) who were unable to detect IFNγ expression in zebrafish infected with *M. marinum* NCIMB 1297 prior to 2 weeks post-infection. Parikka *et al.* (2012) also reported a prolonged, slightly increasing IFNγ response over 7 weeks in zebrafish infected with a low dose of *M. marinum* NCIMB 1297, while fish infected with a high dose had a peak in IFNγ response at 2 weeks, which declined afterwards. TNFα and IFNγ are believed act synergistically in the activation of macrophages, increasing their anti-mycobacterial activity and co-stimulating each other (Kaufmann 2002; Sneller 2002). Furthermore, it has been demonstrated that these two cytokines are important in promotion and function of granulomas (Sneller 2002; Silva Miranda *et al.* 2012). The results of the current study support this hypothesis regarding the results of the previous Chapter 3, where early granuloma formation was detectable between 11 and 14 d.p.i. In humans, immune granuloma formation is normally detectable at 2-3 weeks

after infection (Shaler *et al.* 2012). The results of this study cover only the first 14 days of infection while this latter comment referred to TNFα expression at 28 d.p.i. Thus, there is a need for further investigation of the question of how these important cytokines are expressed over a longer time period and at what time IFNγ becomes significantly induced.

4.5 *Conclusions*

In this study, it was shown for the first time that it is possible to isolate zebrafish leukocytes from individual fish as potential macrophage primary cultures and subsequently infect them with *M. marinum* to investigate macrophage specific gene expression. However, further optimisation has to be carried out to determine the purity of the cultures and perform further *in vitro* infection studies to confirm the results obtained. The further investigation of genes related to the innate immune response such as IL-10 or IL-12 could give insight into the initial response of the macrophage towards the first encounter with a pathogen and how important the communication of the macrophage with other immune cells is for the immune response to *M. tuberculosis*. Based on the *in vivo* housekeeping validation results in Section 4.4.2.3., additional housekeeping gene validations for the *in vitro* experiment has to be performed. In addition, the investigated time period of the *in vitro* infection assay has to be prolonged to 24 or 48 h.p.i. to match the time points of the *in vivo* study and subsequently make accurate comparisons. Nevertheless, such *in vitro* assays show potential for future research.

Furthermore, the pro-inflammatory immune response of adult zebrafish to *M. marinum* NCIMB 1297 and NCIMB 1298 infection was investigated *in vivo* over 14 days for the first time. No inflammatory response was triggered by the injection of *M. marinum* isolate NCIMB 1298 in adult zebrafish which suggests that this isolate is not infective to zebrafish. This is supported by the absence of granuloma formation as described in Chapter 3. In the case of the other isolate, *M. marinum* NCIMB 1297, the

results obtained in this study agreed in most cases with the immune response found in mammals and humans infected with *M. tuberculosis* as well as with initial studies in zebrafish infected with *M. marinum.* With respect to the kinetic profile of the major cytokines associated with mycobacterial immune response, it was possible to establish a chronic, tuberculosis-like infection in adult zebrafish utilizing the *M. marinum* strain NCIMB 1297. By displaying similar kinetics of inflammatory gene expression to those seen in *M. tuberculosis* infection of humans, the zebrafish *M. marinum* model can be applied to the study of human tuberculosis. As demonstrated by Parikka *et al.*, (2012) it is even possible to model the latent stage of the disease (also see Chapter 5), which was not possible with other model organisms except non-human primates. Further studies need to be undertaken using an appropriate infection dose of an *M. marinum* isolate to induce a chronic or latent infection and investigate additional tuberculosis-relevant genes, such as the anti-inflammatory cytokines and further members of the IL-12 family. Such studies need to closely investigate the first months of infection to obtain a better picture of the disease in fish and to provide insights, which could be transferable to studies of mammals including humans.

5 Transcriptome profiling of the head kidney immune response of long-term infected zebrafish under stressful and non-stressful conditions

5.1 *Introduction*

In theory, latent tuberculosis can be considered as an equilibrium state between the host and the bacterium. The host immune response tries to isolate the bacteria within granulomas to prevent an outbreak of the disease, and in response the bacteria enter a dormant state, which protects them from detection and elimination, resulting in a stand-off situation (Ahmad 2010; Lin and Flynn 2010; Patel *et al.* 2011; Ehlers and Schaible 2012; Ernst 2012). In general, this stand-off remains for a lifetime and protects the host from active disease. In some cases, however, the host is not able to maintain this equilibrium; as a result the infection becomes reactivated and the pathogen causes active disease (Flynn and Chan 2001b; Lin and Flynn 2010; Gideon and Flynn 2011).

In reality, the picture is more complex and the dividing line between latent and active disease has been blurred by the discovery of overlapping states, which manifest as a sub-clinical active disease (Gideon and Flynn 2011). The discovery that latently infected hosts exhibiting granulomas at various stages of development, supports the idea that the latent state includes a complex interplay of different states (Lin and Flynn 2010).

One important factor which contributes to the shift in the host-bacterium equilibrium, in favour of the bacterium, is the suppression of the host immune system. In particular, the depletion or lack of Interferon gamma (IFNγ), Tumor Necrosis Factor alpha (TNFα) and / or CD4+ T cells has been associated with the reactivation of tuberculosis. Immunosuppression can have a multiplicity of possible causes. The most

common causes of immunosuppression are: co-infection with another disease *e.g*. HIV; the existence of particular tissue states such as cancer; aging and stress (Gideon and Flynn 2011; Ernst 2012).

Gene expression analyses using microarrays have become an important tool in medical and disease research. Microarrays facilitate a broad overview of the transcriptional activity in an mRNA sample instead of the more limited picture provided by the more traditional single gene or small gene group investigation (Tarca *et al.* 2006; Slonim and Yanai 2009). The principle of the microarray technique, as shown in Figure 5-1, is based on the conversion of messenger (mRNA) into cRNA/ cDNA which is subsequently labelled with different fluorescent dyes to distinguish the experimental groups, e.g. infected versus uninfected, different development stages or different environmental parameters. In the majority of cases, cyanine 3 (Cy3) and Cy5 are used for the analysis. The labelled cRNAs/ cDNAs are subsequently hybridized to the microarray, which results in competitive binding of the differentially labelled targets to the array. By scanning the slide with two different wavelengths, corresponding to the applied dyes, an estimation of the transcription level of each represented genes can be made by comparing the measured intensity in both channels (Goetz and MacKenzie 2008; Zhang *et al.* 2009; Herath 2010; Malakar *et al.* 2013).

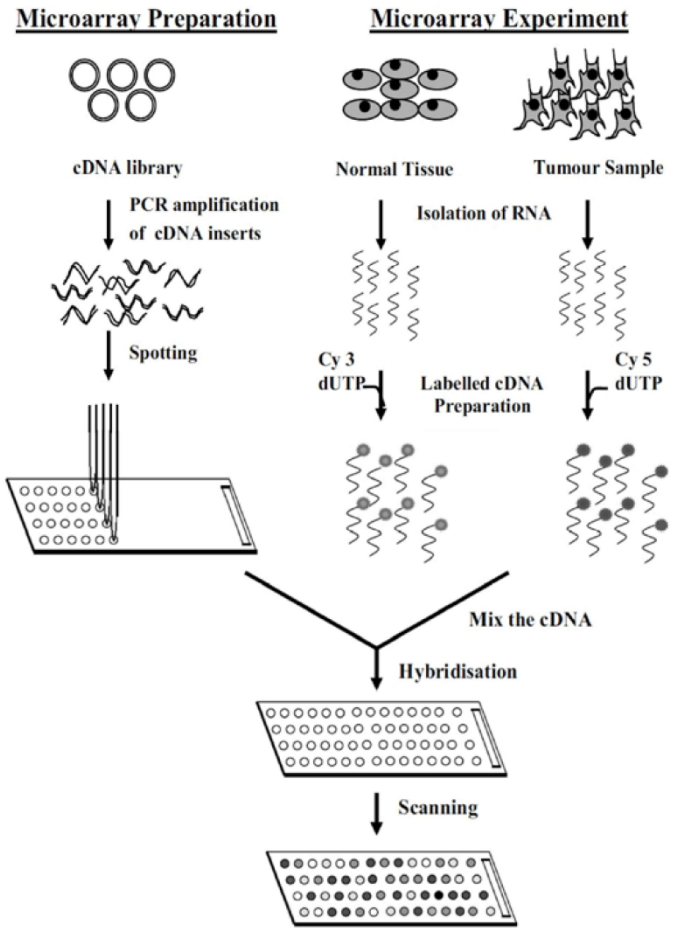

Figure 5-1: Outline of the commercially cDNA microarray technology for gene discovery and transcriptome profiling taken from Malakar *et al.* (2013) including sample and microarray preparation, hybridization and scanning.

In aquaculture and fish biology, transcriptome profiling analyses are performed preferably by two different microarray techniques. In the first method PCR products are used as probes generated from cDNA libraries or clone collections to construct a cDNA microarray. They are subsequently printed onto glass slides or nylon membranes at defined positions, which results in a grid of various spots all comprising the same size and distance from each other. Oligonucleotide arrays, on the other hand, are designed from synthetic single stranded oligonucleotides. Depending on the supplier, these oligonucleotides can be up to 60-mer long, but in general 20-25-mer are the standard (Miller and Maclean 2008; Herath 2010; Malakar *et al.* 2013). In comparison, the cDNA arrays are more economical due to their lower cost and greater flexiblility. The designer of the array has the choice of which cDNA sequences are

spotted onto the slide and can thus design the microarray according to the experimental setup. Oligonucleoide arrays, in contrast, have been designed by a commercial manufacture and are generally comprised of the most abundant genes to give a complete overview of the whole transcriptome (Herath 2010; Malakar *et al.* 2013).

Gene expression profiling using microarray technology has been successfully established in a variety of fish species as shown in Figure 5-2 including zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*).

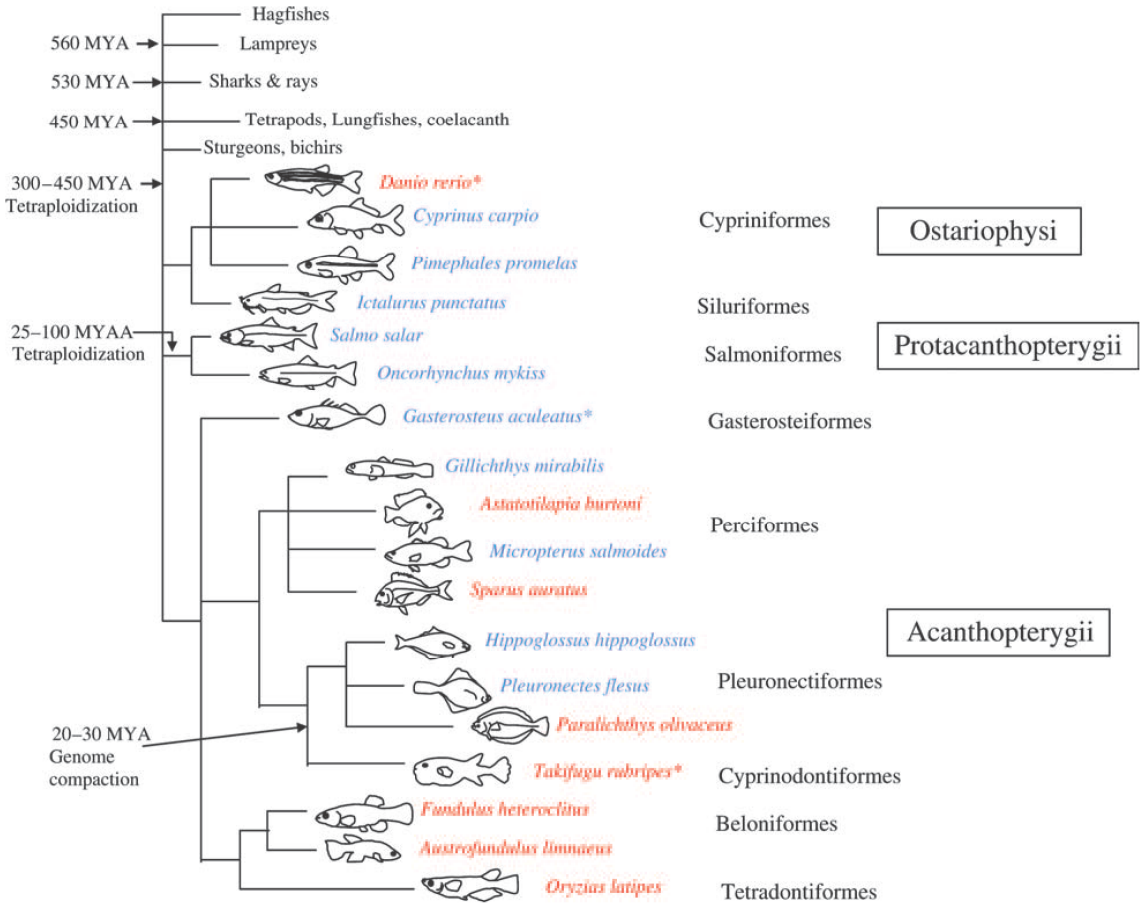

Figure 5-2: Phylogenetic depiction after Nelson (1994) of teleost species for which microarrays have been developed, taken from Miller and Maclean (2008). Microarray platforms for species highlighted in blue are described in Miller and Maclean, (2008) and those in red have been described by other authors. *Species for which complete genome sequences are available contain an asterisk. MYA, millions of years ago.

The studies enable identification of genes related to immunity or disease resistance, response to environmental variation and expression profiling during different development stages or between offspring from different families (Miller and Maclean 2008; Zhang *et al.* 2009; Herath 2010; Malakar *et al.* 2013).

A plethora of microarray analyses have been performed particularly in zebrafish, focusing on embryogenesis (Mathavan *et al.* 2005; Wardle *et al.* 2006; Aanes *et al.* 2013), ecotoxicology (Ung *et al.* 2010; Faltermann *et al.* 2014), development (Zheng *et al.* 2011; Cannon *et al.* 2013) and immunity (Meijer *et al.* 2005; Hegedűs *et al.* 2009; Stockhammer *et al.* 2009; Ordas *et al.* 2011). In the majority of cases, gene expression profiles of experimentally induced diseases have been performed using embryos and have investigated innate immune responses to a pathogen (Stockhammer *et al.* 2009; van der Sar *et al.* 2009; Ordas *et al.* 2011). The few studies conducted thus far on adult fish have focused on a comparison between acute and chronic disease outcomes (Meijer *et al.* 2005; Hegedűs *et al.* 2009; van der Sar *et al.* 2009).

In this Chapter, the zebrafish-*M. marinum in vivo* model has been used to:

1) Establish a *M. marinum* (NCIMB 1297) latent infected population of adult zebrafish
2) Examine the reactivation of the latent disease through stress induction
3) Compare the transcriptomic profile of latent and re-activated disease states in zebrafish

5.2 *Materials and Methods*

5.2.1 Experimental set up

Twelve zebrafish that had been experimentally infected with *M. marinum* NCIMB 1297 and 12 control zebrafish, mock-infected with sterile PBS were utilised. The fish were obtained from the experimental set up described in Chapter 3 and monitored for 6 months post-infection (m.p.i.). After 6 m.p.i., the control and infected fish were further subdivided into 2 separate tanks for each of the treatments of which one was used to perform an additional stress challenge as described in Fig. 5-3.

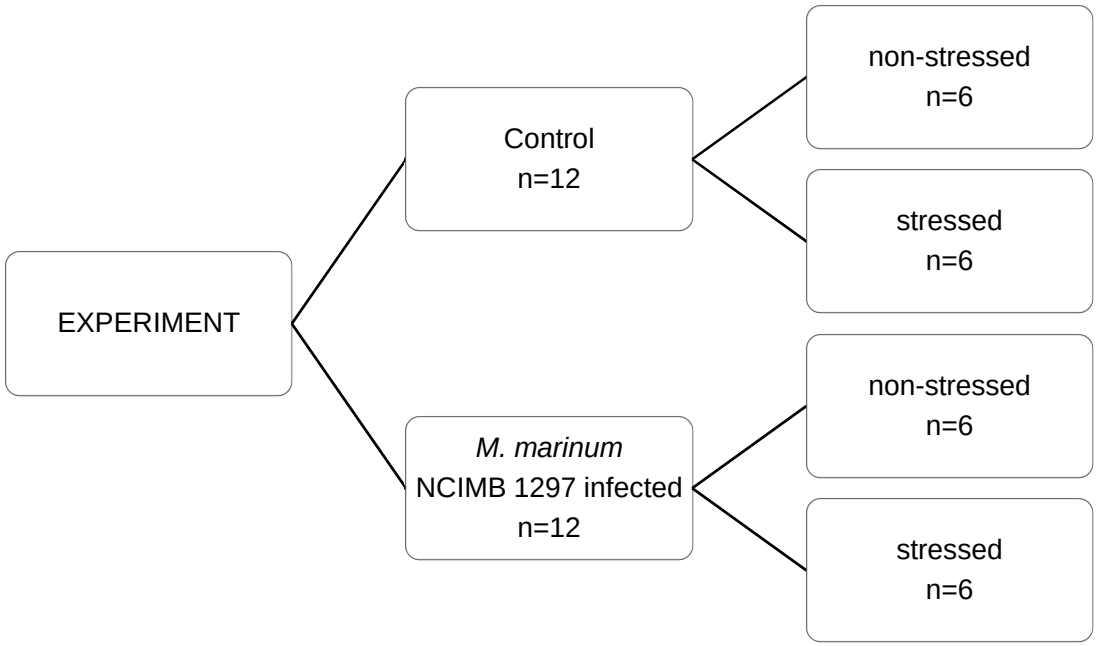

Figure 5-3: Schematic description of the 4 treatment groups employed: control non-stressed, control stressed, *M. marinum* NCIMB 1297 (Mm 1297) infected non-stressed and *M. marinum* NCIMB 1297 infected stressed including the sample size number (n) for each treatment.

The stress challenge was carried out as described in Fig. 5-4. Briefly, the temperature of the tanks was decreased from 28°C to 21°C for 48 h. In addition, the fish were starved to increase stress. After 48 h, the stocking density in the challenged tanks was increased to 6 fish in 200 mL (30 fish/L) for 30 min, before all conditions were re-set to the original conditions. The kidneys were sampled from all fish 2 days

post-challenge. The sampled kidneys were transferred into 100µL RNAlater (Sigma-Aldrich Company Ltd, Dorset, UK) and stored at -80°C for further processing.

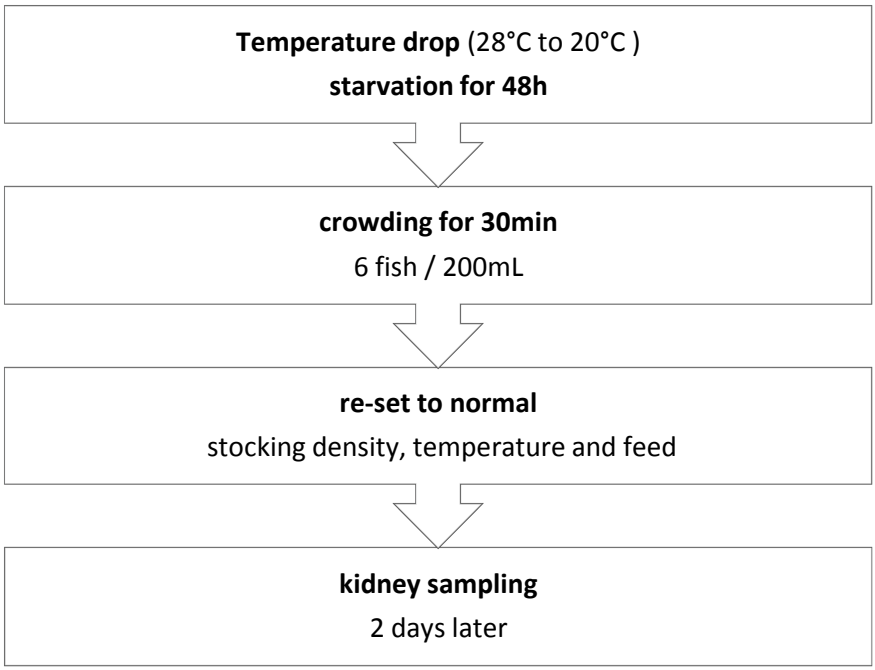

Figure 5-4: Schematic displaying the cascade of applied stress conditions starting with a temperature drop and starvation for 48 h, followed by high density crowding for 30 min. After re-setting of the conditions to normal, the kidneys were sampled 2 days later.

5.2.2 RNA extraction

The frozen kidney samples were taken from the RNA*later* and transferred into new RNase-free 1.5 mL microcentrifuge tubes. The tissue was homogenized in 600 µL RLT buffer using an insulin syringe with a 30-gauge needle (BD bioscience, Oxford, UK) and RNA was extracted with an RNeasy Mini kit (QIAGEN Manchester Ltd, Manchester, UK) following the protocol for purification of total RNA from animal tissue as described in Chapter 2.5.3. Quantification and quality checking of the extracted RNA was determined as described in Chapter 2.5.4.

5.2.3 RNA amplification

RNA was amplified using an Ambion Amino Allyl MessageAmp II Kit (Applied Biosystems, Paisley, UK) according to manufacturer's instructions. The manufacturer's protocol was modified to perform ½ reactions per sample.

5.2.3.1 Reverse Transcription

Five hundred ng RNA were added into a 0.2 mL nuclease-free microfuge tube containing 0.5µL Oligo(dT) primer (100 ng/ µl). The sample was made up to a volume of 6µL using nuclease-free dH_2O, mixed by pipetting and centrifuged briefly at 1000 x g for 5 sec to bring down the sample. Afterwards the tubes were incubated at 70ºC for 10 min on a thermal cycler (Biometra, Göttingen, Germany) with the heated lid on. The master mix for the 1st strand synthesis was prepared in a nuclease-free 0.5 mL microcentrifuge tube at room temperature (~22°C) containing the reagents described in Table 5-1.

Table 5-1: 10x 1st strand buffer master mix protocol adapted from the Ambion Amino Allyl MessageAmp II Kit

REAGENT	µL/reaction
10x 1st strand buffer	1.0
dNTP mix	2.0
RNase Inhibitor	0.5
Arrayscript Enzyme	0.5
Total volume	**4.0**

The 10x 1st strand buffer was pre-warmed to room temperature and checked for any precipitates, which could interfere with the reaction.

The master mix was mixed well and placed on ice till further use. Four µL of the chilled master mix were added to each RNA sample, mixed well, centrifuged briefly and incubated at 42ºC for 2 h in a Stuart Orbital Incubator (Bibby Scientific Limited, Stone, UK). After the incubation, the samples were centrifuged briefly and placed on ice.

5.2.3.2 2nd strand cDNA synthesis

The master mix for the 2nd strand cDNA synthesis was prepared on ice in a nuclease-free 0.5 mL microfuge tube, using following reagents and volumes as described in Table 5-2. The reagents were mixed well by flicking the tube, centrifuged briefly and kept on ice till further use.

Table 5-2: 2nd strand cDNA synthesis master mix protocol adapted from the Ambion Amino Allyl MessageAmp II Kit

REAGENTS	µL/reaction
dH_2O	31.5
10x 2nd strand buffer	5.0
dNTPs	2.0
DNA polymerase	1.0
RNase H	0.5
Total volume	**40.0**

Forty µL of this master mix were added to each sample derived from Section 5.2.3.1. Again the samples were mixed well by flicking and afterwards briefly centrifuged. The samples were incubated at 16ºC on the pre-cooled thermal cycler for 2 h with the heated lid switched off. After the incubation, the samples were centrifuged briefly and kept on ice till further processing.

5.2.3.3 cDNA purification

The cDNA was purified using a QIAquick PCR purification kit (QIAGEN, Manchester, UK), following the manufacturer's PCR purification spin protocol. Prior to purification, 1:250 volume pH indicator I was added to PB buffer. Two hundred and fifty µL of the PB buffer were then mixed with the 50 µL cDNA generated in the previous section. If the colour of the resulting mixture was not yellow, indicating a pH of approximately 7.5, 1-2 µL of 0.3 M sodium acetate were added to adjust the pH. The mixture was transferred to a QIAquick spin column. The column was centrifuged for 1 min at 18000 x g in a microcentrifuge, then placed on a new 2 mL collection tube.

The column was washed with 750 µL PE buffer and centrifuged again for 1 min at 18000 x g. After this step, the column was placed on a new collection tube and centrifuged for 1 min at 18000 x g to dry the membrane of remaining buffer. Afterwards, the QIAquick column was placed into a nuclease-free 1.5 mL microcentrifuge tube and DNA was eluted with 8 µL of pre-warmed (50 – 55°C) nuclease-free water. After 1 min incubation at room temperature (~22°C), the column was centrifuged for 1 min at 18000 x g. The resulting 7 – 8 µL were used immediately for the *in vitro* transcription to synthesize aminoallyl-labelled aRNA as described in Section 5.2.3.4.

5.2.3.4 *In vitro* transcription to synthesise aminoallyl-labelled aRNA

The master mix for the *in vitro* transcription was prepared in a nuclease-free 0.5 mL microcentrifuge tube at room temperature (~22°C), using the reagents and volumes described in Table 5-3.

Table 5-3: *In vitro* transcription master mix protocol adapted from the Ambion Amino Allyl MessageAmp II Kit

Reagent	µL/reaction
aaUTP	1.25
ATP, CTP, GTP mix	6.00
UTP	1.75
10X T7 buffer	2.00
T7 Enzyme mix	2.00
Total volume	**13.00**

The 10x T7 buffer was pre-warmed at room temperature and checked for any precipitates which might interfere with the reaction.

The master mix was mixed well by flicking the tube, centrifuged and placed on ice for further processing. Thirteen µL of the master mix was added to each sample and mixed well by pipetting. After a brief centrifugation step, the samples were incubated for 18 h at 37°C in the Stuart Orbital Incubator.

5.2.3.5 aRNA purification and quantification

Purification of the amplified RNA was performed using the RNA Clean up protocol from the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. The 20 µL of amplified RNA was made up to a volume of 100 µL with nuclease-free water. To that mixture, 350 µL of RLT buffer was added and mixed well by pipetting. Afterwards, 250 µL 95 – 100% ethanol was added to the sample and mixed again. The mixture was transferred to an RNeasy Spin column and centrifuged for 20 sec at 10000 x g. The column was placed in a new 2 mL collection tube and 500 µL RPE buffer were added. The column was washed by centrifugation for 20 sec at 10000 x g. An additional washing step with 500 µL RPE buffer was performed afterwards and the column centrifuged for 2 min at 10000 x g. After that step, the column was placed on a new collection tube again and centrifuged for 1 min at 10000 x g to dry the silica membrane. The amplified RNA was eluted by adding 20 – 50 µL of pre-warmed nuclease-free water directly on to the membrane. After a last centrifugation step for 1 min at 10000 x g, the column was removed and eluted aRNA in the microcentrifuge tube was quantified using a NanoDrop. The amplified RNA was stored at -70°C for further use.

5.2.4 aRNA dye labelling, coupling and purification

The individual amplified RNA (aRNA) samples were labelled with Cy3^{TM} dye (GE Healthcare, Little Chalfont, UK). Subsequently, a pool of all individual samples was created and labelled with Cy5^{TM}dye (GE Healthcare). The labelling of the aRNA was performed according to the manufacturer's protocol with minor adjustment. Briefly, 2 µL of the two dye samples were pre-diluted in ultra-pure dimethyl sulphoxide (DMSO) (Sigma–Aldrich) and 2 – 3 µg of each aRNA were transferred to individual new 1.5 mL microcentrifuge tubes and adjusted with nuclease-free water to a final volume of 10µL. The samples were incubated at 70°C for 2 min on a dri-block (Techne, Bibby Scientific Limited) and then cooled down to room temperature (22°C).

Each sample was mixed with 3 µL of 0.5 m $NaHCO_3$ (Sigma-Aldrich), followed by the addition of 1.33 µL of the appropriate Cy dye (Cy3 for individual samples and Cy5 for the pooled samples). After a gentle mix by pipetting, the samples were placed in a Stuart Orbital Incubator and incubated at 25°C for 1 h in the dark.

Illustra AutoSeq G-50 Dye terminator spin columns (GE Healthcare) were used to purify the aRNA and remove uncoupled dye. The purifications were performed by first removing the bottom closure of the column and placing it in a 2 mL collection tubes. The column was pre-spun in an IEC MicroMax centrifuge at 2000 x g for 1 min and placed on a new collection tube before the dye-aRNA mixture was carefully transferred onto the gel bed. The loaded columns were centrifuged at 2000 x g for 1 min and afterwards the spin columns were removed from the tubes and discarded. The quantity of labelled aRNA was determined by NanoDrop and quality was determined by an agarose gel electrophoresis. Subsequently the gel was scanned on a Typhoon scanner (GE Healthcare) to visualize the labelled aRNA. Samples were stored at -70°C until further analysis.

5.2.5 Microarray hybridisation

The Agilent zebrafish V3 microarray comprising 44000 oligo probes (Agilent, Wokingham, UK) was used for this study. In total, 4 slides, each containing 4 arrays, were utilised for this study giving the opportunity to investigate 4 individual fish samples for the 4 created states of infection challenge (control, control + stressed, infected, infected + stressed). Each array was hybridised with individual Cy3-coupled samples and the Cy5-coupled pool.

For each sample, 825 ng of Cy3 and Cy5- coupled pool were transferred to a new microcentrifuge tube and adjusted to a total volume of 35 µL with nuclease-free water. Subsequently, 11.0 µL 10X Blocking Agent, 2.2 µL 25X Fragmentation Buffer and 6.8 µL nuclease-free water were added to each sample and each mixture was incubated at 60°C on a heat block for 30 min in the dark. After this incubation, 57 µL

2X GEx Hybridisation Buffer and 3 µL nuclease-free water were added to each sample. The samples were centrifuged at 15000 x g for 1 min and placed on ice until they were loaded onto the arrays.

A clean 4 array gasket glass slide (Agilent) was placed into an Agilent SureHyb chamber base with the labels facing upwards and aligned with the rectangular section of the chamber base. The gaskets were loaded with the hybridisation samples in a random order.

After carefully loading the samples into the gaskets, one of the microarray slides was placed onto the gasket slide, facing the active side of the array downwards. After verification of the proper alignment of the two slides, the SureHyb chamber cover was placed onto the slides. The loading of the hybridisation sample onto the gaskets and subsequent covering with the microarray was repeated for the remaining three microarray slides. All 4 SureHyb chambers containing the microarray slides were transferred into the hybridisation oven (Agilent) and hybridised at 65° for 17 h. The oven rotator was set to a rotation speed of 10 rpm for an even distribution of the 2X GEx Hybridisation Buffer HI-RPM.

5.2.6 Microarray washing

After the overnight hybridisation, the microarrays were washed according to the Agilent protocol. Washing buffers were pre-made. Briefly, 2 mL 10% Triton X-102 was added to Gene Expression Wash Buffer 1 and 2. The array-gaskets were removed from the SureHyb chamber, placed into an EasyDip rack and transferred into an EasyDip jar (Canemco, Canada) containing wash buffer 1. Subsequently, the rack was placed into a new EasyDip jar containing wash buffer 1 and incubated for 1 min at room temperature. Afterwards, the rack washed for 1 min with wash buffer 2, pre-warmed to 37°C and aliquoted into another EasyDip jar. After carefully removing the slide rack from the washing buffer, the microarray slides were placed in a slide box and centrifuged at 500 x g for 6 min. The slides were kept in the dark till scanning.

5.2.7 Microarray scanning

The microarray slides were scanned with an Axon 4200A Scanner in combination with the GenePix Pro 6.1 software. The final values of the hardware settings comprised 35% for the laser power for both the green and the red laser for all four scanned arrays as described in Table 5-4. The photomultiplier tube (PMT) settings varied depending on the laser colour and the scanned array and are described in Table 5-4.

Table 5-4: Final hardware settings for the laser power and photomultiplier tube (PMT) for each of the scanned microarrays

Slide Nr	Scan			
	Green Laser		Red Laser	
	% power	PMT	% power	PMT
4	35	405	35	383
5	35	431	35	386
6	35	453	35	383
7	35	414	35	376

5.2.8 Data processing

The raw scanned data were imported into Agilent Feature Extraction Software (v9.5) (Agilent Technologies) and prepared for Genespring analysis. After importing the extracted data into Genespring GX Multi-Omic Analysis (v12.6.1) (Agilent Technologies, Inc.), a sequence of quality filters was applied as described in Table 5-5 to ensure the highest quality and consistency of targets on which the statistical analysis was performed.

Differences between the four treatment groups were determined using a 2-way ANOVA ($p \leq 0.05$) without employing the false discovery rate correction. It has been shown that the false discovery rate correction step may often remove key gene targets and is therefore considered to be over conservative (personal communication Dr. J. Taggart and Prof. J. E. Bron, Institute of Aquaculture, Herath, 2010). A fold-change cut-off value of ±1.3 was set for the final interpretation of the results.

Table 5-5: Summary of the applied filter on the extracted data in Genespring

Filter applied:	Percent/ States	Description
ControlType = 0	in 100%	Removal of all control probes
gIsSaturated = 0 and rIsSaturated = 0	in 75% of 4x16 state system	Removal of all unsaturated signals
gIsFeatNonUnifOL = 0 and rIsFeatNonUnifOL = 0		Removal of all non-uniform signals
gIsFeatPopnOL = 0 and rsFeatPopnOL = 0		Removal of all population outliers
gIsPosAndSignif = 1 and rIsPosAndSignif = 1		Removal of all negative (non-positive) and non-significant signals

For further analysis of the functional breakdown of the significantly expressed targets, two gene lists were created for each of the two treatments, infected and infected*stress, containing the top 1000 targets sorted by the lowest p-value. These two lists were exported into Excel where the targets with no annotation or un-known identity were removed. In addition, the gene lists were further subdivided into lists comprising either all up or down-regulated genes for each of the two treatments, resulting in a total of four gene lists for functional analysis. The freely available BioEdit© Sequence Alignment Editor was used to create FASTA format files from the four excel lists which were subsequently uploaded into KEGG KAAS for KEGG pathway analysis (*www.genome.jp/tools/kaas/)*. The pathway analysis was carried out to determine to which molecular interaction and reaction network the significant targets belonged to.

5.2.9 Validation of microarray detected by RT-PCR

A number of key genes, which had been significantly differentially expressed relative to expression in the control group in the microarray analysis, were selected to validate the microarray. Validation was performed by quantitative real-time PCR (qRT-PCR) as described in Chapter 2.5.8. Prior to qRT-PCR analysis, 400 ng of the

originally extracted zebrafish kidney RNA were converted into cDNA as described in Chapter 2.5.5. A total amount of 2.5 ng was used for each sample per PCR reaction and the expression of each chosen gene was measured in triplicate.

A total number of 8 key immune genes were chosen to validate the microarray. The target sequence and primary accession number provided by Agilent were used to create specific primer pairs. The primary accession number was entered into NCBI primer BLAST (NCBI, 2013) which set up was changed to find specific primers for *Danio rerio* within the first 300 bp of the target sequences. The resulting primer pairs were tested for their target specificity by BLASTing them against the target sequence again. In addition to the 8 immune genes, the expression of three house-keeping genes was measured. Two of these house-keeping genes, elongation factor 1 alpha and ribosomal protein L13a were obtained from Chapter 4. The other gene, metastasis associated 1 family member 2 (mta2), was a so-called flatliner, originated from the microarray analysis with hardly any detectable expression changes. The primer sequences, their product sizes and annealing temperatures are summarised in Table 5-6.

Table 5-6: Summary of the primer pairs used for validation including the gene name of the target sequence, specific primer name, primer sequence, resulting product size and annealing temperature. The first eight genes comprise the key immune genes, followed by the three house-keeping genes.

Gene	Primer name	Primer sequence (5'-> 3')	Product size (bp)	Annealing temperature (°C)
Complement component 3c3	CC3c3-F1	TGTGGAGGCGCAGGATTATT	130	62.0
	CC3c3-R1	TGTAAGACTCTGGTATTTTGCTCCA		
Chemokine (C-X-C motif) ligand 12a	CXCl12a-F1	CGCCATTCATGCACCGATTT	143	63.2
	CXCl12a-R1	TGACTTGGAAGGGGCAGTTG		
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH-F1	AGAGGCTTCTCACAAACGAGG	200	65.0
	GAPDH-R1	CTTTCCATGGGTGGAGTCGTA		
Interferon gamma	IFNγ-VoiF	CTTTCCAGGCAAGAGTGCAGA	101	59.3
	IFNγ-VoiR	TCAGCTCAAACAAAGCCTTTCG		
Interleukin 1 beta	IL-1β-F4	TGAAGTCACCATAGCTCCAAAA	148	61.5
	IL-1β-R4	CTGCGAAGTCCACATCTCCA		
Nuclear Factor of kappa Light polypeptide gene enhancer in B-cells inhibitor, alpha a	NFkBIAa-F1	AGAGGAATATGAGACGCGGG	116	65.0
	NFkBIAa-R1	CTCCGTCCTCGGTGACTACT		
Transforming Growth Factor beta	TGFb-F1	AGTCAGTGATGGATGTGTAAAGGA	134	64.4
	TGFb-R1	GATGAATGAAGCGGCTCCCA		
Tumor Necrosis Factor alpha	TNFα-F5	ACTCGATTTGACTTAACAAAACCTT	197	62.0
	TNFα-R5	AATGATCCCAAACACCCTCCA		
Elongation factor 1 alpha	ELF1a-F2	TGCCTTCGTCCCAATTTCAG	101	57.5
	ELF1a-R2	TACCCTCCTTGCGCTCAATC		
Ribosomal protein L13a	RPL13a-F1	TCTGGAGGACTGTAAGAGGTATGC	148	57.5
	RPL13a-R1	AGACGCACAATCTTGAGAGCAG		
Metastasis associated 1 family, member 2	MTA2-F1	TGTCTGAGAGGGGCTTCGAT	186	64.9
	MTA2-R1	TATATCCTCGCGTGTGCTGG		

5.3 *Results*

5.3.1 Microarray analysis

After statistical analysis, a total number of 12412 targets were significantly differentially expressed ($p<0.05$) compared to the non-stressed and non-infected (NS/NI) control group.

The fish which were exposed solely to stress (S/NI group) exhibited a total number of 6986 significantly differentially expressed targets of which 4454 were unique to this fish group. In the non-stressed and infected (NS/I) fish a total number of 6074 targets were differentially expressed of which 3358 were unique to this particular group. The fish which were exposed to both treatments, stress and infection (S/I), expressed a total number of 3115 targets differentially expressed, of which 1300 were unique to this group. A summary of the target numbers uniquely expressed and shared between two or all three groups is shown as a Venn diagram Fig. 5-5.

To get a better overview, the differentially expressed targets for each treatment group, were plotted as a volcano plot with the p-value plotted against fold change. From the 6074 significantly expressed targets in the infected group, 3598 were down- and 2476 were up-regulated. In the stress x infection group, 1893 targets were down and 1222 targets were up-regulated. The fish exposed solely to stress displayed an up-regulation of 3420 targets while 3566 targets were down-regulated. By comparing the three volcano plots the differences in number and direction of regulation relative to unstressed and uninfected fish can be observed (Fig. 5-6).

This chapter focuses on the effects of stress on long-term infection and therefore only the gene lists for infected and stress x infection were further investigated.

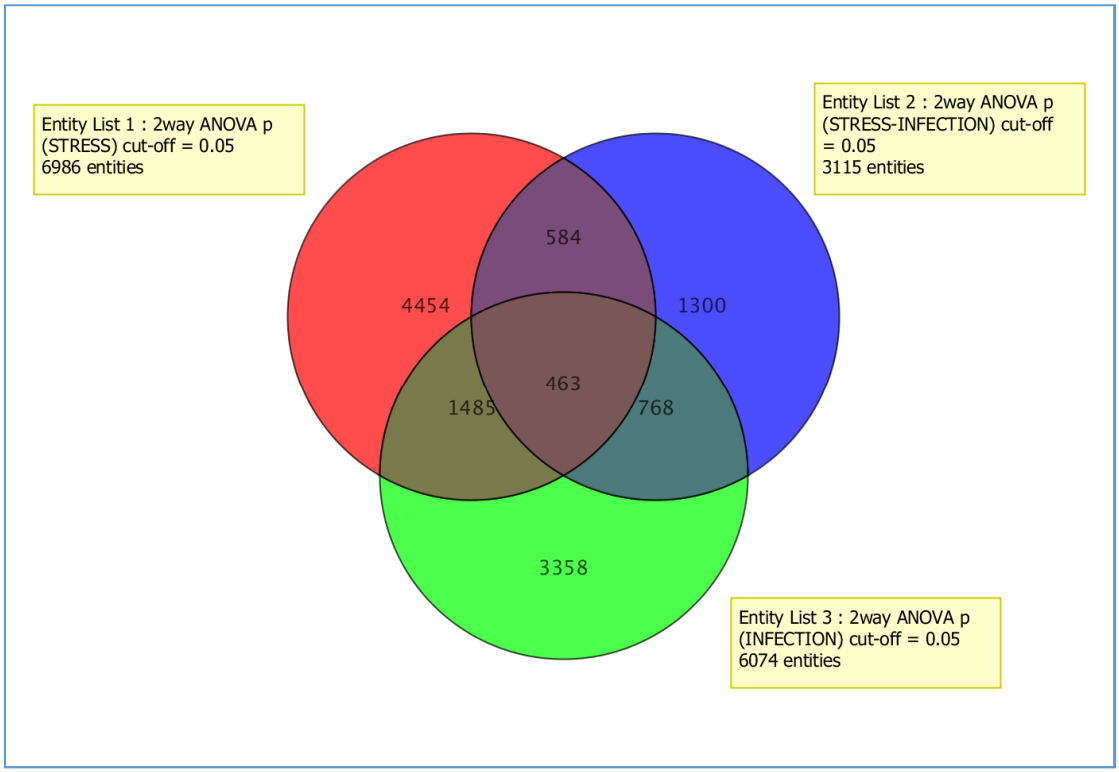

Figure 5-5: Venn diagram summarising the number of significantly expressed genes for each of the three treatments. Green: unstressed and infected, red: stressed and uninfected, blue: stressed and infected. The significantly differentially expressed genes were determined relative to the uninfected and unstressed control group by applying a 2-way-ANOVA with a p value cut off of 0.05.

The top 1000 targets of each of the of the ANOVA lists of infection and stress x infection were further analysed to establish identity, relationships and function of differentially regulated targets. After removal of unidentified targets which included unknown, no identity or un-named targets, a total number of 918 targets remained for the infection gene list and 912 targets for the stress x infection list. Further separation of the lists into up- and down-regulated targets revealed that 259 targets were up- and 659 gene were down- regulated in the infection list while in the stress x infection gene list 368 targets were up- and 546 targets were down-regulated.

The four resulting lists, infection up- or down-regulated and stress x infection up- or down-regulated, were transformed into FASTA files using BioEdit sequence alignment editor (Hall, 1994) and subsequently analysed for matches of existing pathway elements and functional annotation using KAAS (KEGG Automatic Annotation Server) (Moriya *et al.*, 2007) (Fig. 5-7).

The matched KEGG targets were further sub-divided according to their involvement in immune-related functions such as environmental signal processing, cell growth and death, immune system and tuberculosis. In addition, the four original target lists were manually analysed using internet-based tools such as iHOP, Genecards, NCBI or EMBL-EBI to detect immune-related targets which had been missed by the KAAS-KEGG analysis.

The final results comprise of a combination of both analyses, categorised and visualised as heat maps according to their involvement in innate and / or adaptive immunity and cell death such as apoptosis.

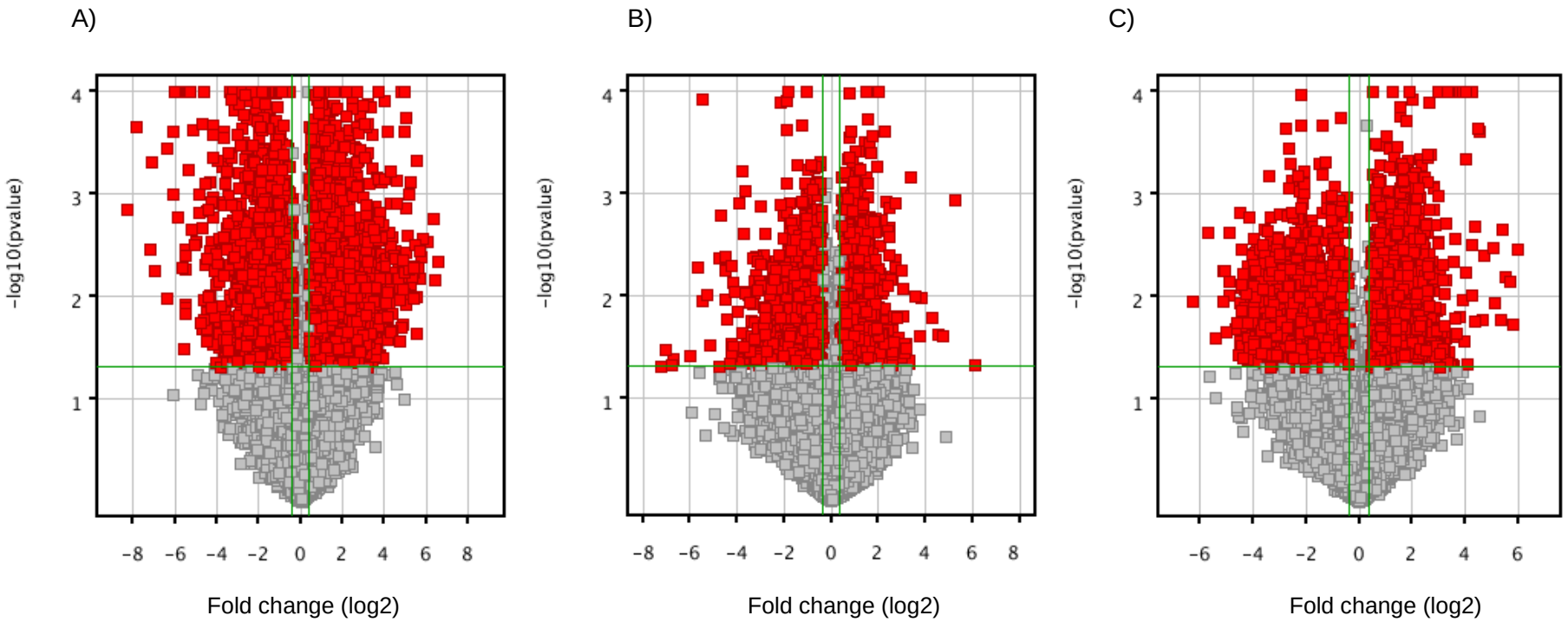

Figure 5-6: Volcano plots of gene expression relative to uninfected and unstressed zebrafish for: A) unstressed and infected, B) stressed and uninfected and C) stressed and infected zebrafish. Significantly expressed genes with a p-value < 0.05 and a fold change >±1.3 are illustrated as red squares while the genes which do not fall into these categories are coloured grey. The green bars illustrate the threshold for the p-value and fold change.

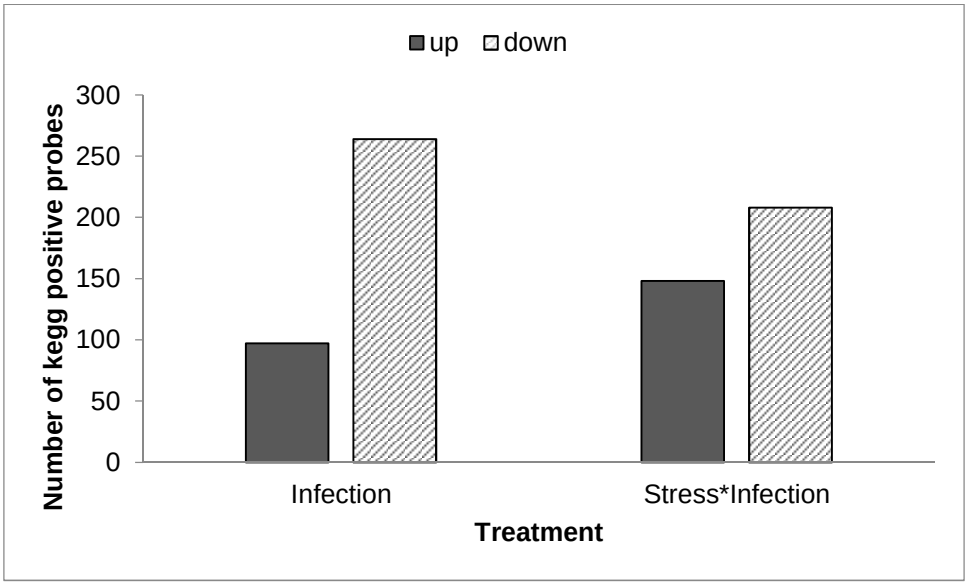

Figure 5-7: Comparison of KAAS positive targets for infected and stress x infected fish. The top 1000 up and down-regulated targets derived from 2-way ANOVA analysis in Genespring were uploaded into KAAS-KEGG database and the number of positive hits are displayed as a bar graph according to treatment and direction of regulation.

5.3.2 Innate immune response

A large number of immune and apoptosis-related genes were significantly differentially expressed in the infected and stress x infected fish. The immune-response related genes were further subdivided into innate and adaptive immune response, although some genes shared categories and were therefore included in multiple heat maps (Fig. 5-8 A – 5-13 B).

The innate host response for infected but unstressed fish comprised a number of significantly differentially regulated acute phase proteins such as coagulation factor IIIb (F3b) and V (F5), fibronectin 1 (FN1) and serum amyloid A (SAA) which were positively induced (Fig. 5-8 A) while other acute phase proteins including alpha-2-antiplasmin, complement factor 6 (C6) and C3c, protein C (PROC) and serine proteinase inhibitor, clade D, member 1 (SERPIND1) displayed negative regulation (Fig. 5-8 B). A number of chemokines and cytokines were significantly up-regulated such as TNFα and its induced proteins TNFα induced protein 2 (TNFAIP2) and TNFAIP3-interacting protein 1-like (TNFAIP3), chemokine (C-X-C-motif) ligand 11-like

(CXCL 11l), Interleukin 8 while others were down-regulated *e.g.* CXCL 12a. Several genes which promote a positive inflammatory response were significantly up-regulated including caspase recruitment domain family, member 9 (CARD9), dual specificity phosphatase 2 (DUSP2) and mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3) as well as genes related to respiratory burst and reactive oxygen production such as neutrophil cytosolic factor 4 (NCF4), inducible nitric oxide synthase 2b (NOS2b) and thioredoxin (TXN). In addition, genes encoding catenin (cadherin-associated protein), interleukin-1 receptor-associated kinase 3 (IRAK3), interferon regulatory factor 10 (IRF10) and matrix metalloproteinase 14b (MMP14b) and were also significant up-regulated. Further down-regulated genes included anti-inflammatory mediator SMAD6 and leukocyte differentiation and proliferation related genes such as Rho GTPase activating protein 21b and Phosphatidylethanolamine binding protein 1 (PEBP1).

A)

S	I	S_I	p value	Description
1.98	5.36	3.46	0.000115	Alpha-2-macroglobulin receptor (probe probe seq 1)
1.73	4.78	3.21	0.000277	Alpha-2-macroglobulin receptor (probe probe seq 2)
1.92	7.86	1.35	0.000190	Caspase recruitment domain family, member 9 (card9) (probe probe seq 1)
1.80	7.21	1.35	0.000128	Caspase recruitment domain family, member 9 (card9) (probe probe seq 2)
2.13	2.83	3.58	0.000830	Catenin (Cadherin-associated protein)
3.86	62.10	2.06	0.000280	Chemokine (C-X-C motif) ligand 11 like
2.12	5.35	5.80	0.000223	Chondroitin sulfate proteoglycan 5a
1.09	11.28	1.27	0.000133	Coagulation factor IIIb (f3b)
2.00	4.34	4.24	0.001067	Coagulation factor V (f5)
1.19	1.51	1.65	0.002283	Cylindromatosis (turban tumor syndrome), a
1.06	6.81	2.18	0.000013	Dual specificity phosphatase 2 (dusp2) (probe seq 1)
1.04	6.00	1.97	0.000029	Dual specificity phosphatase 2 (dusp2) (probe seq 2)
-1.20	5.30	1.25	0.000006	Dual specificity phosphatase 2 (dusp2) (probe seq 3)
1.19	3.65	1.37	0.000472	Dual specificity phosphatase 5 (dusp5)
-1.47	3.49	1.09	0.000126	Fibronectin 1 (fn1)
1.41	4.51	1.18	0.000267	Heparanase (hpse)
1.61	2.73	3.66	0.000212	Interferon regulatory factor 10 (irf10)
2.54	25.63	2.03	0.000173	Interleukin 8 - Cyprinus carpio (Common carp), partial (94%)
1.58	4.34	1.18	0.000291	Interleukin-1 receptor-associated kinase 3 (IRAK3)
-1.19	5.25	1.02	0.000219	Matrix metalloproteinase 14b (membrane-inserted) (mmp14b)
1.93	4.57	2.87	0.000056	Mitogen-activated protein kinase-activated protein kinase 3 (mapkapk3)
3.52	29.76	2.17	0.000009	Myxovirus (influenza virus) resistance C (mxc)
-1.10	2.64	1.38	0.000060	Neutrophil cytosolic factor 4
1.53	42.62	1.63	0.000018	Nitric oxide synthase 2b, inducible (nos2b)
-1.11	1.58	1.24	0.000202	Potassium channel tetramerisation domain containing 13 (kctd13)
-1.00	2.91	1.91	0.000308	Protein tyrosine phosphatase, receptor type, E
1.75	1.69	4.57	0.000353	Rac GTPase-activating protein 1
2.23	315.21	3.39	0.000393	Serum amyloid A (saa) (probe seq 1)
1.47	46.62	1.52	0.000079	Serum amyloid A (saa) (probe seq 2)
2.19	12.62	3.04	0.000011	Thioredoxin (txn) (probe seq 1)
1.78	9.96	2.44	0.000004	Thioredoxin (txn) (probe seq 2)
-1.08	4.99	-1.17	0.000245	TNFAIP3-interacting protein 1-like
1.59	67.68	1.54	0.000024	Tumor necrosis factor a (TNF superfamily, member 2) (tnfa)
1.68	15.00	-1.04	0.000475	Tumor necrosis factor, alpha-induced protein 2
1.26	26.11	1.66	0.000206	Wilms tumor 1b (wt1b) (probe seq 1)
-1.18	10.39	1.18	0.000269	Wilms tumor 1b (wt1b) (probe seq 2)

B)

S	I	S_I	p value	Description
-2.44	-27.67	-10.18	0.00015	Alpha-2-antiplasmin (probe seq 1)
-5.80	-50.68	-60.44	0.00010	Alpha-2-antiplasmin (probe seq 2)
-4.92	-30.34	-32.06	0.00008	Alpha-2-antiplasmin (probe seq 3)
-1.53	-2.55	-2.11	0.00197	Chemokine (C-X-C motif) ligand 12a (stromal cell-derived factor 1) (cxcl12a)
-1.47	-3.37	-2.96	0.00224	Claudin
1.22	-3.40	-1.75	0.00147	Claudin 7b (cldn7b) (probe seq 1)
1.54	-3.40	-1.16	0.00204	Claudin 7b (cldn7b) (probe seq 2)
-2.59	-2.42	-4.29	0.00112	Complement component 6 (c6)
7.54	-9.11	-3.00	0.00088	Complement component c3c (c3c)
-1.39	-2.07	-1.80	0.00160	Guanine nucleotide binding protein (G protein), gamma 7 (gng7)
1.41	-3.13	-1.71	0.00043	LIM domain kinase 2 (limk2) (probe seq 1)
1.20	-3.54	-1.58	0.00006	LIM domain kinase 2 (limk2) (probe seq 2)
1.41	-2.29	-1.08	0.00177	Mitochondrial antiviral signaling protein (mavs), nuclear gene encoding mitochondrial protein
-1.47	-3.42	-2.69	0.00002	Neuronal calcium sensor 1a (ncs1a)
-1.26	-9.47	-2.18	0.00009	Phosphatidylethanolamine binding protein 1 (pebp1)
-1.25	-7.55	-2.25	0.00064	Protein C (inactivator of coagulation factors Va and VIIIa) (probe seq 1)
-2.09	-11.64	-4.36	0.00005	Protein C (inactivator of coagulation factors Va and VIIIa) (probe seq 2)
1.09	-1.52	-1.05	0.00011	Ras homolog gene family, member Gc (rhogc)
-1.30	-1.73	-3.81	0.00001	Rho GTPase activating protein 21b
-1.23	-6.84	-1.99	0.00130	Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1 (serpind1) (probe seq 1)
-5.80	-50.68	-60.44	0.00010	Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1 (serpind1) (probe seq 2)
-1.73	-2.11	-7.66	0.00006	SMAD family member 6a (smad6a)

Figure 5-8 A + B: Heat maps of significantly, differentially regulated innate immune related genes in the head kidney of adult zebrafish. The columns represent the significant A) up-regulated or B) down-regulated targets for the (I) long-term infected zebrafish compared to the non-stressed and non-infected control group. Additionally, the other two treatments, (S) stress x non-infected and (S_I) stress x infection and the p value for the investigated group are visualised. Regulation of genes is visualised in blue for down-regulation and red for up-regulation whereas the numbers indicate the fold change of the gene relative to unstressed uninfected controls.

In the stressed x infected fish, the microarray analysis revealed significant differential expression of innate immune genes (Figure 5-9 A + B). As with the infected fish, a number of acute phase proteins were up-regulated including serum amyloid A (SAA) and complement component c3a (C3a) or down-regulated such as complement component 3 (C3), complement component c3c (C3c), complement factor B (CFB). Further, coagulation factors were significantly expressed including coagulation factor IIIb (F3b), glycoprotein Ib beta (GP1bb), integrin alpha 2b (ITGA2b) and protein C (PROC). Innate response cytokines and chemokines were significantly up-regulated including C-C-motif chemokine 8 (CCL8), chemokine (C-X-C-motif) ligand 11-like (CXCL11l), interleukin 1 beta (IL-1β), interleukin 8 (IL-8), tumor necrosis factor alpha (TNFα) and beta (TNF b). Down-regulated transcripts included chemokine (C-X-C-motif) ligand c1c (CXCL-c1c), CXCL14, CCL-C20g, which is a member of the novel chemokine subfamily CX and transforming growth factor beta 3 (TGFb3). In addition, TNFAIP2 and TNFAIP3-interacting protein 1-like were down-regulated. A number of important receptor signalling pathway genes, inflammatory regulation and responding genes were significantly up-regulated including DUSP2, interferon regulatory factor 1b (IRF1b), IL-15 receptor alpha chain (IL-15ra), matrix metalloproteinase 9 (mmp9), MAPKAPK3, NOS2b, receptor-interacting serine-theonine kinase 2 (RIPK2), suppressor of cytokine signalling 1 (SOCS1) and thioredoxin (Fig. 5-9 A) while others were down-regulated, including interferon-induced protein 44 (IFI44), mitogen-activated protein kinase kinase kinase 8 (MAP3K8), myristoylated alanine-rich C-kinase substrate (MARCKS)-like 1b and nuclear factor of kappa light polypeptide gene enhance in B cells 2 p49/p100 (NFKB2) (Fig.5-9 B). Notably, some immune-related genes were significantly up- and down- regulated in the stressed x infected fish including CARD9, catenin, myxovirus resistance c (MXC), nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor alpha a (NFKBIAA) and b (NFKBIAB) as well as transferrin-a . This phenomenon was only observed in this particular fish and in no other treatment group.

A)

S_UI	US_I	S_I	p value	Description
1.92	7.86	1.35	0.000007	Caspase recruitment domain family, member 9 (card9) (probe seq 1)
1.80	7.21	1.35	0.000008	Caspase recruitment domain family, member 9 (card9) (probe seq 2)
1.53	1.83	1.23	0.006780	Catenin (cadherin-associated protein), alpha (ctnna) (probe seq 1)
-1.88	-1.77	1.02	0.009940	C-C motif chemokine 8
3.86	62.10	2.06	0.000017	Chemokine (C-X-C motif) ligand 11 like (cxcl11l)
1.09	11.28	1.27	0.000402	Coagulation factor IIIb (f3b)
3.73	19.97	1.04	0.000170	Complement component c3a
-1.20	5.30	1.25	0.000533	Dual specificity phosphatase 2
1.76	2.80	1.53	0.000355	Guanine nucleotide binding protein (G protein), gamma 12a (gng12a)
1.41	4.51	1.18	0.000030	Heparanase (hpse)
4.19	11.82	3.18	0.000600	Interferon regulatory factor 1b (irf1b) (probe seq 1)
2.63	6.06	1.61	0.000178	Interferon regulatory factor 1b (irf1b) (probe seq 2)
15.22	20.71	6.06	0.000108	Interleukin 1, beta (il1b) (probe seq 1)
8.19	11.56	2.43	0.000036	Interleukin 1, beta (il1b) (probe seq 2)
2.54	25.63	2.03	0.000049	Interleukin 8 - Cyprinus carpio (Common carp), partial (94%)
1.20	2.63	1.16	0.005423	IL-15 receptor alpha chain (il-15ra)
9.60	23.16	2.61	0.000348	Matrix metalloproteinase 9 (mmp9)
1.43	4.46	1.04	0.000169	Mitogen-activated protein kinase-activated protein kinase 3 (mapkapk3)
3.52	29.76	2.17	0.000009	Myxovirus (influenza virus) resistance C (mxc) (probe seq 1)
1.53	42.62	1.63	0.000025	Nitric oxide synthase 2b, inducible (nos2b)
2.14	4.52	1.18	0.000002	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a (nfkbiaa) (probe seq 1)
1.49	2.06	1.31	0.001535	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 1)
1.57	2.02	1.35	0.000632	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 2)
1.64	1.95	1.62	0.003546	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 3)
-1.69	-1.44	1.11	0.006242	Phospholipase A2, group IVA (cytosolic, calcium-dependent)
2.17	3.31	1.69	0.000554	Protein tyrosine phosphatase type IVA, member 3
3.72	8.97	1.63	0.000294	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1b (ptprz1b)
3.77	11.23	3.13	0.000760	Receptor-interacting serine-threonine kinase 2 (ripk2) (probe seq 1)
5.09	12.70	1.60	0.001919	Receptor-interacting serine-threonine kinase 2 (ripk2) (probe seq 2)
1.47	46.62	1.52	0.000091	Serum amyloid A (saa)
1.27	3.50	1.06	0.000717	Suppressor of cytokine signaling 1 (socs1)
2.19	12.62	3.04	0.000122	Thioredoxin (txn) (probe seq 1)
1.78	9.96	2.44	0.000059	Thioredoxin (txn) (probe seq 2)
5.81	59.09	1.30	0.000101	Transferrin-a (tfa) (probe seq 1)
3.33	24.16	1.18	0.000138	Transferrin-a (tfa) (probe seq 2)
2.39	17.10	1.00	0.000082	Transferrin-a (tfa) (probe seq 3)
1.59	67.68	1.54	0.000020	Tumor necrosis factor a (TNF superfamily, member 2) (tnfa)
3.83	7.50	2.19	0.000080	Tumor necrosis factor b (TNF superfamily, member 2) (tnfb)
1.26	26.11	1.66	0.000815	Wilms tumor 1b (wt1b)

B)

S_UI	US_I	S_I	p value	Description
1.39	8.21	-1.32	0.000098	Caspase recruitment domain family, member 9 (card9) (probe seq 3)
1.21	1.68	-1.05	0.010199	Catenin (cadherin-associated protein), alpha (ctnna) (probe seq 2)
1.37	1.68	-1.11	0.006772	Catenin (cadherin-associated protein), alpha 2 (ctnna2)
1.36	5.90	-1.16	0.000005	Chemokine (C-X-C motif) ligand C1c (cxcl-c1c) (probe seq 1)
1.31	5.83	-1.23	0.000025	Chemokine (C-X-C motif) ligand C1c (cxcl-c1c) (probe seq 2)
-1.78	-4.37	-1.42	0.001135	Chemokine (C-X-C motif) ligand 14 (cxcl14) (probe seq 1)
-1.67	-4.22	-1.39	0.002010	Chemokine (C-X-C motif) ligand 14 (cxcl14) (probe seq 2)
-1.71	-3.70	-1.31	0.001978	Chemokine (C-X-C motif) ligand 14 (cxcl14) (probe seq 3)
-1.69	-3.88	-1.29	0.001991	Chemokine (C-X-C motif) ligand 14 (cxcl14) (probe seq 4)
4.02	18.46	-1.02	0.002117	Complement component 3
5.39	8.70	-1.26	0.000508	Complement component c3c (c3c)
-1.59	2.69	-2.61	0.007861	Component factor B
-4.01	-6.34	-2.75	0.000013	Ectonucleoside triphosphate diphosphohydrolase 2 a.1 (entpd2a.1)
-1.19	-2.53	-1.09	0.000490	Exodeoxyribonuclease 7 small subunit - Gluconobacter oxydans (Gluconobacter suboxydans)
-2.21	-2.36	-1.80	0.001868	Glycoprotein Ib (platelet), beta polypeptide (gp1bb) (probe seq 1)
-2.18	-2.18	-1.77	0.003690	Glycoprotein Ib (platelet), beta polypeptide (gp1bb) (probe seq 2)
1.01	2.25	-1.17	0.006531	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-5
-2.30	-1.96	-1.11	0.001139	Integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41B) (itga2b)
1.40	6.25	-1.12	0.000384	Interferon-induced protein 44 (IFI44)
1.34	6.14	-1.18	0.000400	Lysozyme g-like 1 (lygl1)
1.50	3.09	-1.14	0.000004	MARCKS-like 1b (probe seq 1)
1.71	3.52	-1.06	0.000000	MARCKS-like 1b (probe seq 2)
-1.56	2.95	-2.53	0.010952	Mitogen-activated protein kinase kinase kinase 8
4.14	64.01	-1.08	0.000037	Myxovirus (influenza virus) resistance C (mxc) (probe seq 2)
3.94	13.42	-1.31	0.000405	Myxovirus (influenza virus) resistance F
-2.78	-3.08	-1.82	0.000668	Novel chemokine subfamily CX chemokine CCL-C20g
1.06	1.85	-1.12	0.000122	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100 (nfkb2)
1.41	2.83	-1.15	0.000163	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a (nfkbiaa) (probe seq 2)
1.16	1.76	-1.27	0.003489	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 4)
-1.06	8.71	-1.79	0.000962	Prostaglandin-endoperoxide synthase 2b (ptgs2b)
-1.29	-1.25	-1.22	0.005583	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1 (pin1)
-2.09	-11.64	-4.36	0.006160	Protein C (inactivator of coagulation factors Va and VIIIa) (proc)
-1.08	4.99	-1.17	0.000104	TNFAIP3-interacting protein 1-like
6.46	69.13	-1.30	0.000213	Transferrin-a (tfa) (probe seq 4)
5.65	45.29	-1.14	0.000655	Transferrin-a (tfa) (probe seq 5)
-1.36	3.66	-2.28	0.000059	Transforming growth factor, beta 3 (tgfb3)
1.34	9.21	-1.51	0.000051	Tumor necrosis factor, alpha-induced protein 2 (probe seq 1)
1.68	15.00	-1.04	0.000011	Tumor necrosis factor, alpha-induced protein 2 (probe seq 2)
1.21	2.62	-1.64	0.005648	Ubiquitin thioesterase (CYLD) (probe seq 1)
1.29	2.26	-1.36	0.002967	Ubiquitin thioesterase (CYLD) (probe seq 2)
-1.87	-1.42	-1.38	0.005628	Wiskott-Aldrich syndrome (eczema-thrombocytopenia) a

Figure 5-9 A + B: Heat maps of significantly, differentially regulated innate immune related genes in the head kidney of adult zebrafish. The columns represent the significant A) up-regulated or B) down-regulated targets for the (S_I) stress x infected zebrafish compared to the non-stressed and non-infected control group. Additionally, the other two treatments, (S) stress x non-infected and (I) infection and the p value for the investigated group are also visualised. Regulation of genes is visualised in blue for down-regulation and red for up-regulated genes, whereas the numbers indicate the fold change of the gene relative to unstressed uninfected controls.

A number of innate immune related genes were significantly differentially expressed in both treatments *e.g*. C3c, CARD9, CXCL 11, F3b, IL-8, MAPKAP3, NOS2b, PROC, SAA, thioredoxin and TNFα. By comparing the fold change of these genes according to each treatment, it was clear that in the infection-only fish these genes consistently showed higher expression than in the stressed x infected fish.

5.3.3 Adaptive immunity

A large number of adaptive immunity-related genes were significantly differentially expressed in the infected fish compared to the control fish including genes involved in antigen-presenting and processing such as ATP-binding cassette sub-family B (MDR/TAP) member 3 like 1 (ABCB3l1), beta-2-microglobulin (B2M), calreticulin-like (CALRl), cathepsin L 1 a (CTSl1a) and V-akt murine thymoma viral oncogene homolog 2 like (AKT2l) (Fig. 5-10 A + B). Further, B and T cell receptor pathway related genes including protein kinase C, theta (PRKCq) and ras homolog gene family, member ae (RHOAE) were significantly up-regulated, while other genes of these pathways such as CD81 antigen (TAPA1), V-akt murine thymoma viral oncogene homolog 2, like (AKT2l) and V-Ha-ras Harvey rat sarcoma viral oncogene homolog a (HRASA) were down-regulated. In addition, a number of genes encoding natural killer cell-mediated cytotoxicity and cytokines mediating between adaptive and innate immunity, as well as their inducing or induced genes, were significantly up-regulated including fas ligand TNF superfamily member 6 (FASLG), guanylate binding protein 2, interferon regulatory factor 10 (IRF10), interferon gamma 1-2 (IFNγ 1-2), TNFα, tumor necrosis factor receptor superfamily member 9a (TNFRSF9a) and TNFAIP2 (Fig. 5-10 A).

A)

S_UI	US_I	S_I	p value	Description
1.44	12.82	1.19	0.00051	Allograft inflammatory factor 1-like (aif1l)
1.34	3.53	1.75	0.00039	ATP-binding cassette, sub-family B (MDR/TAP), member 3 like 1 (abcb3l1)
1.20	2.72	1.59	0.00019	Beta-2-microglobulin (b2m), transcript variant 1,
2.68	4.10	5.55	0.00078	Calreticulin like (calrl)
2.13	2.83	3.58	0.00083	Catenin (Cadherin-associated protein), alpha
-1.16	1.43	1.59	0.00207	Fas ligand (TNF superfamily, member 6) (faslg)
1.32	3.48	2.48	0.00004	Guanylate binding protein 2
1.61	2.73	3.66	0.00021	Interferon regulatory factor 10 (irf10)
2.02	22.07	3.92	0.00058	Interferon, gamma 1-2 (ifng1-2)
1.28	3.64	3.49	0.00023	Protein kinase C, theta (prkcq)
1.10	1.32	1.45	0.00069	Ras homolog gene family, member Ac (rhoac)
1.02	1.95	1.39	0.00108	Ras homolog gene family, member Ae (rhoae)
1.59	67.68	1.54	0.00002	Tumor necrosis factor a (TNF superfamily, member 2) (tnfa)
1.74	11.44	1.99	0.00002	Tumor necrosis factor receptor superfamily, member 9a (tnfrsf9a)
1.68	15.00	-1.04	0.00048	Tumor necrosis factor, alpha-induced protein 2

B)

S_UI	US_I	S_I	p value	Description
-1.72	-3.56	-3.14	0.000804	Cathepsin L, 1 a (ctsl1a) (probe seq 1)
1.05	-2.96	-1.16	0.002339	Cathepsin L, 1 a (ctsl1a) (probe seq 2)
1.05	-2.73	-1.33	0.001768	Cathepsin L, 1 a (ctsl1a) (probe seq 3)
-2.05	-6.76	-2.48	0.000030	CD81 antigen (TAPA1) (probe seq 1)
-1.54	-6.65	-1.54	0.000134	CD81 antigen (TAPA1) (probe seq 2)
-1.47	-3.37	-2.96	0.002236	Claudin
1.22	-3.40	-1.75	0.001472	Claudin 7b (cldn7b) (probe seq 1)
1.54	-3.40	-1.16	0.002037	Claudin 7b (cldn7b) (probe seq 2)
1.33	-3.18	-1.67	0.000005	Immunoglobulin-like domain containing receptor 1b (ildr1b)
-1.42	-2.10	-2.42	0.000009	NEDD4 binding protein 2 (n4bp2)
-1.92	-14.11	-4.21	0.000118	Nuclear receptor subfamily 2, group F, member 6b (nr2f6b)
1.98	-3.95	-1.07	0.000675	V-akt murine thymoma viral oncogene homolog 2, like (akt2l)
-1.46	-2.09	-1.72	0.001643	V-Ha-ras Harvey rat sarcoma viral oncogene homolog a (hrasa)

Figure 5-10 A + B: Heat map of significantly, differentially regulated adaptive immune related genes in the head kidney of adult zebrafish. The columns represent the significant A) up-regulated or B) down-regulated targets for the (I) long-term infected zebrafish compared to the non-stressed and non-infected control group. Additionally, the other two treatments, (S) stress x non-infected and (S_I) stress x infection and the p value for the investigated group are visualised. Regulation of genes is visualised in blue for down-regulation and red for up-regulated whereas the numbers indicate the fold change of the gene relative to unstressed uninfected controls.

As with the infected-only fish, the stressed x infected fish displayed a large number of adaptive immune related genes which were significantly differentially expressed (Fig. 5-11 A + B). Besides genes with innate and adaptive immunity-related functions, which were significantly differentially regulated such as catenin, IL-15ra, M3K8, NFKB2, NFKBIAA, NFKBIAB, TNFα, TNF b and TNFIAP2, a number of specific adaptive immune response genes were expressed. These specific adaptive immune genes included members of the antigen presentation by the major histocompatibility complex (MHC) class I such as ATP-binding cassette, subfamily B, member 3 like 1 (ABCB3l1) which was up-regulated (Fig.5-11 A) while other members including beta-2-microglobulin (B2M), proteasome activator subunit 1 (PSME1) and TAP binding protein (TAPSIN) were down-regulated (Fig 5-11 B). Genes belonging to the B and T cell receptor signalling pathways were significantly differentially expressed including CD8 alpha1, CD8 antigen beta, CD81, ST6 beta-galactosamide alpha-2,6-sialyltransferase (ST6GAL1), T cell immune regulator 1 ATPase H^+transporting lysosomal V0 subunit A3, TNFRSF9a and tumor necrosis factor superfamily member 13b (TNFSF13b). In addition, FASLG, which is part of the natural killer cell mediated cytotoxicity, immunoglobulin superfamily containing leucine-rich repeat 2 (ISLR2) and phospholipase A2, group IVA which is part of the Fc epsilon RI signalling pathway were significantly up-regulated (Fig. 5-11 A).

A)

S_UI	US_I	S_I	p value	Description
1.44	12.82	1.19	0.000147	Allograft inflammatory factor 1-like (aif1l)
1.34	3.53	1.75	0.007998	ATP-binding cassette, sub-family B (MDR/TAP), member 3 like 1 (abcb3l1)
1.53	1.83	1.23	0.006780	Catenin (cadherin-associated protein), alpha (ctnna) (probe seq 1)
2.33	10.76	1.39	0.001655	CD8 alpha1
4.26	17.69	2.74	0.004834	CD8B antigen, beta
1.27	2.39	1.26	0.009340	Fas (TNF receptor superfamily, member 6)
1.20	2.63	1.16	0.005423	IL-15 receptor alpha chain (il-15ra)
-1.45	-2.66	1.38	0.000100	Immunoglobulin superfamily containing leucine-rich repeat 2 (islr2)
2.14	4.52	1.18	0.000002	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a (nfkbiaa) (probe seq 1)
1.64	1.95	1.62	0.003546	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 1)
1.57	2.02	1.35	0.000632	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 2)
1.49	2.06	1.31	0.001535	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 3)
-1.69	-1.44	1.11	0.006242	Phospholipase A2, group IVA (cytosolic, calcium-dependent)
1.59	67.68	1.54	0.000020	Tumor necrosis factor a (TNF superfamily, member 2) (tnfa)
3.83	7.50	2.19	0.000080	Tumor necrosis factor b (TNF superfamily, member 2) (tnfb)
1.74	11.44	1.99	0.000051	Tumor necrosis factor receptor superfamily, member 9a (tnfrsf9a)

B)

S_UI	US_I	S_I	p value	Description
1.11	2.47	-1.01	0.00584	Beta-2-microglobulin (b2m), transcript variant 2
1.21	1.68	-1.05	0.01020	Catenin (cadherin-associated protein), alpha (ctnna) (probe seq 2)
1.37	1.68	-1.11	0.00677	Catenin (cadherin-associated protein), alpha 2 (ctnna2)
-2.05	-6.76	-2.48	0.00018	CD81 molecule (probe seq 1)
-1.54	-6.65	-1.54	0.00013	CD81 molecule (probe seq 2)
-1.19	-2.53	-1.09	0.00049	Exodeoxyribonuclease 7 small subunit - Gluconobacter oxydans (Gluconobacter suboxydans), partial (19%)
-1.56	2.95	-2.53	0.01095	Mitogen-activated protein kinase kinase kinase 8
1.06	1.85	-1.12	0.00012	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100 (nfkb2)
1.41	2.83	-1.15	0.00016	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a (nfkbiaa) (probe seq 2)
1.16	1.76	-1.27	0.00349	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 4)
1.55	139.92	-1.03	0.00022	Osteoclast stimulatory transmembrane protein (ocstamp)
-1.00	1.66	-1.10	0.00682	Proteasome activator subunit 1 (psme1)
-2.11	-4.26	-1.45	0.00080	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1 (st6gal1)
-1.30	1.45	-1.97	0.00875	TAP binding protein (tapasin)
-1.24	1.47	-1.67	0.01066	TAP binding protein (tapbp)
1.00	2.90	-1.28	0.00092	T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3
-1.14	3.65	-1.41	0.00053	Tumor necrosis factor (ligand) superfamily, member 13b (tnfsf13b) (probe seq 1)
-1.10	3.62	-1.23	0.00245	Tumor necrosis factor (ligand) superfamily, member 13b (tnfsf13b) (probe seq 2)
1.68	15.00	-1.04	0.00001	Tumor necrosis factor, alpha-induced protein 2 (probe seq 1)
1.34	9.21	-1.51	0.00005	Tumor necrosis factor, alpha-induced protein 2 (probe seq 2)

Figure 5-11 A + B: Heat maps of significantly, differentially regulated adapted immune related genes in the head kidney of adult zebrafish. The columns represent the significant A) up-regulated or B) down-regulated targets for the (S_I) stress*infected zebrafish compared to the non-stressed and non-infected control group. Additionally, the other two treatments, (S) stress*non-infected and (I) infection and the p value for the investigated group are visualised. Regulation of genes is visualised in blue for down-regulation and red for up-regulation whereas the numbers indicate the fold change of the gene relative to unstressed uninfected controls.

5.3.4 Apoptosis and cell death-associated genes

All infected fish displayed significant, differential expression of cell death associated genes. In the infected-only fish group, the number of significantly differentially expressed genes was lower than in the stress x infected fish. Cell death encoding genes in the infected fish comprised baculoviral IAP repeat-containing 7 (BIRC7), CARD9, FASLG, IRAK3, prostaglandin I2 synthase (PTGIS) and TNFα and TNF b were significantly up-regulated (Fig. 5-12 A). Genes including AKT2l, calpain 1 (CAPN1a), members of the cytochrome p450 family 2 (CYP2AA1, CYP2P6, CYP2P7) and uveal autoantigen with coiled-coil domains and ankyrin repeats were significantly down-regulated (Fig. 5-12 B).

In the stress x infected fish, a number of specific cell death-related genes were significantly differentially expressed in addition to those already described for innate and adaptive immunity such as CARD9, FASLG, IL-1β, IRAK3, NFKBIAA, NFKBIAB, TNFα, TNF b and TNFAIP2 (Fig. 5-13 A + B). Specific cell death encoding genes which were significantly up-regulated included cytochrome c somatic b (CYCSb), growth arrest and DNA-damage-inducible alpha a (GADD45AA), MOB kinase activator 1 Bb (MOB1Bb) and optineurin (OPTN) (Fig. 5-13 A). On the other hand, significant down-regulated genes included GULP engulfment adaptor PTB domain containing 1, phorbol-12-myristate-13-acetate-induced protein 1, scotin, sestrin 1 (SESN1) and tripartite motif containing 35-9 (TRIM35-9) (Fig. 5-13 B). Two genes displayed similar unusual regulation pattern as previously described in the innate and adaptive responses of stress x infected fish. DNA-damage regulated autophagy modulator 1 (DRAM1) and growth arrest and DNA-damage-inducible beta a (GADD45BA) showed both, up- and down-regulation.

A)

S_UI	US_I	S_I	p value	Description
1.07	1.79	2.96	0.002259	Baculoviral IAP repeat-containing 7 (birc7)
1.92	7.86	1.35	0.000190	Caspase recruitment domain family, member 9 (card9) (probe seq 1)
1.80	7.21	1.35	0.000128	Caspase recruitment domain family, member 9 (card9) (probe seq 2)
-1.16	1.43	1.59	0.002074	Fas ligand (TNF superfamily, member 6) (faslg)
1.58	4.34	1.18	0.000291	Interleukin-1 receptor-associated kinase 3
-1.19	3.20	2.16	0.000329	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits (gnptab)
-1.03	16.18	-1.01	0.000138	Prostaglandin I2 (prostacyclin) synthase (ptgis)
1.59	67.68	1.54	0.000024	Tumor necrosis factor a (TNF superfamily, member 2) (tnfa)
1.68	15.00	-1.04	0.000475	Tumor necrosis factor, alpha-induced protein 2

B)

S_UI	US_I	S_I	p value	Description
1.11	-2.66	-1.45	0.00088	Calpain 1, (mu/I) large subunit a (capn1a)
-1.15	-3.96	-2.54	0.00001	Cytochrome P450, family 2, subfamily AA, polypeptide 1 (cyp2aa1)
-1.26	-4.98	-2.70	0.00012	Cytochrome P450, family 2, subfamily P, polypeptide 6 (cyp2p6)
-2.36	-3.00	-4.32	0.00006	Cytochrome P450, family 2, subfamily P, polypeptide 7 (cyp2p7)
-1.25	-1.58	-1.82	0.00208	Stromal antigen 2a (stag2a)
1.45	-1.89	-1.47	0.00004	Uveal autoantigen with coiled-coil domains and ankyrin repeats
1.98	-3.95	-1.07	0.00067	V-akt murine thymoma viral oncogene homolog 2, like (akt2l)

Figure 5-12 A + B: Heat maps of significantly, differentially regulated cell death related genes in the head kidney of adult zebrafish. The columns represent the significant A) up-regulated or B) down-regulated targets for the (I) long-term infected zebrafish compared to the non-stressed and non-infected control group. Additionally, the other two treatments, (S) stress*non-infected and (S_I) stress*infection and the p value for the investigated group are visualised. Regulation of genes is visualised in blue for down-regulation and red for up-regulation whereas the numbers indicate the fold change of the gene relative to unstressed uninfected controls.

A)

S_UI	US_I	S_I	p value	Description
1.92	7.86	1.35	0.000007	Caspase recruitment domain family, member 9 (card9) (probe seq 1)
1.80	7.21	1.35	0.000008	Caspase recruitment domain family, member 9 (card9) (probe seq 2)
44.83	12.42	5.08	0.006157	Cyclin E1 (ccne1)
1.88	2.22	1.47	0.010785	Cytochrome c, somatic b (cycsb)
1.25	7.85	1.15	0.000394	DNA-damage regulated autophagy modulator 1 (dram1) (probe seq 1)
1.27	2.39	1.26	0.009340	Fas (TNF receptor superfamily, member 6)
3.55	3.25	2.58	0.006490	Growth arrest and DNA-damage-inducible, alpha, a (gadd45aa)
1.61	1.79	1.26	0.007040	Growth arrest and DNA-damage-inducible, beta a (gadd45ba) (probe seq 1)
15.22	20.71	6.06	0.000108	Interleukin 1, beta (il1b) (probe seq 1)
8.19	11.56	2.43	0.000036	Interleukin 1, beta (il1b) (probe seq 2)
1.58	4.34	1.18	0.000007	Interleukin-1 receptor-associated kinase 3
1.02	3.09	1.00	0.000075	MOB kinase activator 1Bb (mob1bb)
2.14	4.52	1.18	0.000002	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a (nfkbiaa) (probe seq 1)
1.64	1.95	1.62	0.003546	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 1)
1.57	2.02	1.35	0.000632	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 2)
1.49	2.06	1.31	0.001535	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 3)
1.32	1.76	1.16	0.000255	Optineurin (optn)
1.59	67.68	1.54	0.000020	Tumor necrosis factor a (TNF superfamily, member 2)
3.83	7.50	2.19	0.000080	Tumor necrosis factor b (TNF superfamily, member 2) (tnfb)

B)

S_UI	US_I	S_I	p value	Description
1.39	8.21	-1.32	0.00010	Caspase recruitment domain family, member 9 (card9)
1.16	6.48	-1.20	0.00015	DNA-damage regulated autophagy modulator 1 (dram1) (probe seq 2)
1.60	1.89	-1.07	0.00001	Growth arrest and DNA-damage-inducible, beta a (gadd45ba) (probe seq 2)
-3.27	-7.98	-2.07	0.00053	GULP, engulfment adaptor PTB domain containing 1
1.06	1.85	-1.12	0.00012	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100 (nfkb2)
1.41	2.83	-1.15	0.00016	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a (nfkbiaa) (probe seq 2)
1.16	1.76	-1.27	0.00349	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (nfkbiab) (probe seq 4)
1.40	3.70	-1.04	0.00016	Phorbol-12-myristate-13-acetate-induced protein 1
-1.10	2.74	-1.38	0.00604	Scotin
-1.40	-2.54	-1.05	0.00030	Sestrin 1 (sesn1)
-1.39	-2.66	-1.25	0.00003	Sphingosine-1-phosphate receptor 5a (s1pr5a)
-8.39	-6.17	-1.55	0.00062	Tripartite motif containing 35-9 (trim35-9)
1.68	15.00	-1.04	0.00001	Tumor necrosis factor, alpha-induced protein 2 (probe seq 1)
1.34	9.21	-1.51	0.00005	Tumor necrosis factor, alpha-induced protein 2 (probe seq 2)

Figure 5-13 A + B: Heat maps of significantly, differentially regulated cell death related genes in the head kidney of adult zebrafish. The columns represent the significant A) up-regulated or B) down-regulated targets for the (S_I) stress*infected zebrafish compared to the non-stressed and non-infected control group. Additionally, the other two treatments, (S) stress*non-infected and (I) infection and the p value for the investigated group are visualised. Regulation of genes is visualised in blue for down-regulation and red for up-regulation whereas the numbers indicate the fold change of the gene relative to unstressed uninfected controls.

5.3.5 Validation by qRT-PCR

Eight significantly differentially expressed targets were chosen to validate the microarray analysis by qRT-PCR (Fig. 5-14). Seven genes were selected due to their involvement the in immune response (C3c, CXCL 12a, IL-1β, IFNγ, NFkBa, TGF b and TNFα) and their significant expression in one or both of the treatments, infection and stress x infection. In addition, GADPH was chosen due to its role in metabolism. The majority of genes chosen for validation displayed similar patterns of expression to those seen in the microarray analysis. The fold change obtained from the qRT-PCR is compared to that seen in microarray results in Table 5-14.

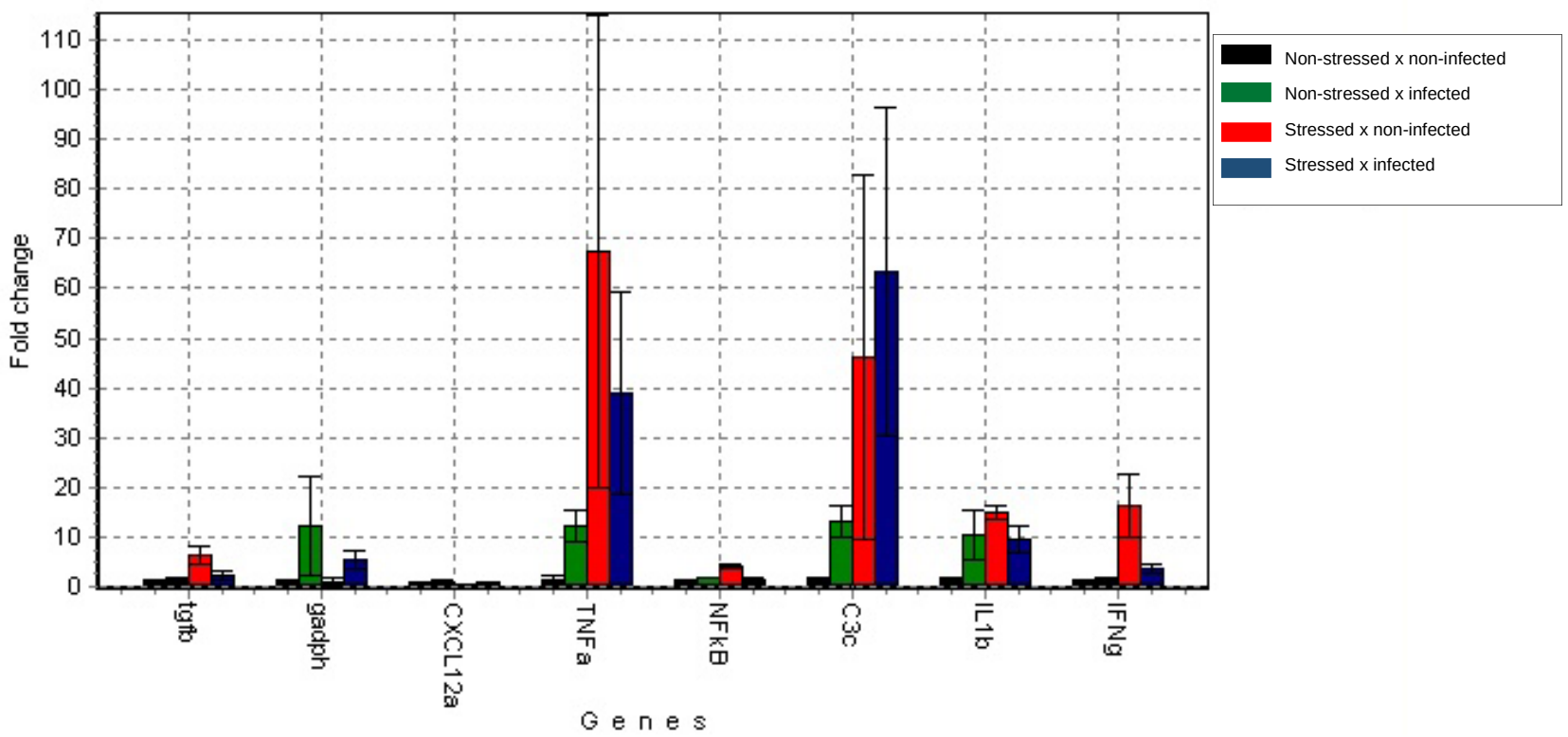

Figure 5-14: Validation of microarray by quantitative RT-PCR (qRT-PCR) of selected immune-related genes. Expression levels of eight significantly differentially regulated immune-related genes including standard error (SE) derived from the microarray analysis were measured by qRT-PCR and normalised using three housekeeping genes.

Table 5-7: Microarray fold change of the validated genes compared to the qRT-PCR results. Significantly differentially expressed (p<0.05) target genes in the microarray are highlighted in grey.

Gene	Microarray FC			qRT-PCR FC		
	US_I	S_UI	S_I	US_I	S_UI	S_I
Complement component 3c3	-9.11	7.54	-3.00	46.15	12.92	63.42
Chemokine (C-X-C motif) ligand 12a	-2.55	-1.53	-2.11	0.51	1.04	0.83
Glyceraldehyde-3-Phosphate Dehydrogenase	-4.24	1.4	-1.64	1.1	12.43	5.61
Interferon gamma	22.07	2.02	3.92	16.46	1.4	3.79
Interleukin 1 beta	20.71	15.22	6.06	14.91	10.45	9.28
Nuclear Factor of kappa Light polypeptide gene enhancer in B-cells inhibitor, alpha a	4.52	2.14	1.18	4.06	1.63	1.28
Transforming Growth Factor beta	-1.4	3.7	-2.3	6.3	1.3	2.44
Tumor Necrosis Factor alpha	67.68	1.59	1.54	67.37	12.33	38.83

5.1 Discussion

In this Chapter, asymptomatic adult zebrafish carrying long-term infections with *M. marinum* were exposed to a stressful environment, in this case a combination of starvation, high crowding density and low ambient water temperature. This exposure was expected to trigger reactivation of the latent, underlying disease which is characterised by lack of clinical signs despite infection.

Gene expression profiles were compared between infected and stress x infected fish to determine significant differences which might aid the understanding of latency and reactivation. The analysis focused on immune and cell death related genes because these genes are important in containing the bacteria and maintaining the equilibrium between host immune response and bacterial interactions (Chan and Flynn 2004; Lin and Flynn 2010; Gideon and Flynn 2011; Ernst 2012).

A decision was made not to pool tissue samples, which allowed generic and individual variability to be assessed. Re-activation of diseases strongly depends on the individual host response and therefore a high variability was observed in the microarray experiment (data not shown) and subsequently confirmed by the exhibition of large standard errors in the qRT-PCR validation. The results obtained from the qRT-PCR validation in general agreed with the findings from the microarray. Differences may result from a number of causes. Data normalisation is performed using two different methods. While expression in qRT-PCR is normalised relative to housekeeping genes, which are thought to be unaffected by the treatments, the microarray data is normalised relative to a pooled reference control (Morey *et al.* 2006; Thallinger *et al.* 2012). Furthermore the targeted region might differ between microarray and qRT-PCR. Microarray probes may fail to recognise possible splice variants while primer pairs for qRT-PCR normally only recognise a subset of the splice variants (Thallinger *et al.* 2012). Although it is recommended to have the primer pairs designed to recognise the same target zone as the microarray probe (Thallinger *et al.*

2012), this is not always possible and some primer pairs are chosen simply to allow specific binding to the RNA template.

The templates for the microarray and qRT-PCR also differ. In a microarray experiment the extracted RNA is purified to only contain mRNA. This purification step is missing in qRT-PCR which allows the binding of the primers to tRNA and rRNA. Furthermore, the efficiencies of the reverse transcriptases used to convert the RNA to cDNA might differ between microarray and qRT-PCR (Morey *et al.* 2006; Thallinger *et al.* 2012).

To date, no universal validation methods for microarray have been proposed and qRT-PCR is still the most commonly used and favoured approach (Thallinger *et al.* 2012).

5.1.1 Latent/ sub-clinically infected fish

Most transcriptional analyses of the zebrafish *M. marinum* model as well as with for pathogens such as *Salmonella*, have been performed using embryos (Stockhammer *et al.* 2009; van der Sar *et al.* 2009; Ordas *et al.* 2011)**.** These studies lack the adaptive immune input which is important in latency and re-activation of tuberculosis and the results of these studies are therefore difficult to compare to this one. Studies performed on adult zebrafish focus in general on the chronic disease outcome where fish were sacrificed after 2 months following the display of clinical signs (Meijer *et al.* 2005; Hegedűs *et al.* 2009; van der Sar *et al.* 2009). Although these data might not be suitable for discussion of the transcriptomic analysis in latent infected zebrafish from this study, they might provide some reference for the results obtained from the zebrafish with re-activated disease.

The adult zebrafish, analysed in this microarray experiment, were infected with *M. marinum* NCIMB 1297 for 26 weeks (6.5 months). During this time they showed no clinical signs which led to the conclusion that these fish had successfully developed a sub-clinical or latent disease stage.

The successful infection of these fish was confirmed in Chapter 3 where the granuloma formation driven by the experimental injection of zebrafish with *M. marinum* was monitored. Granulomas are not only the hallmark of tuberculosis but also for granulomatous inflammation, a specific outcome of chronic inflammation (Williams and Williams 1983; Kumar *et al.* 2012). Repair of damaged tissue and cells and its replacement is one characteristic of chronic inflammation. When this is not possible, the inflammatory cascade continues. Therefore, it is possible that the gene expression patterns of latent and subclinical tuberculosis are similar to the ones found in chronic inflammation.

Elevation of acute phase proteins such as serum amyloid A (SAA) as well as other acute phase proteins and coagulation factor V and IIIb confirm that the zebrafish were still responding to a persisting inflammatory stimulus. SAA is an accepted marker for inflammation, both acute and chronic (Salazar *et al.* 2001; López-Campos *et al.* 2012) and is used to diagnose tuberculosis infections (Agranoff *et al.* 2006; Walzl *et al.* 2011).

M. tuberculosis infections in humans and non-human primates normally pass into a latent or sub-chronically infection which is characterised by the absence of clinical signs despite activation of the immune response. Additionally, an equilibrium between pro- and anti-inflammatory cytokines is detectable (Mack *et al.* 2009; Young *et al.* 2009; Lin and Flynn 2010; Gideon and Flynn 2011; Patel *et al.* 2011; Ernst 2012). The host immune response and the bacterium reside in a standoff state where infection is controlled by the immune response but viable bacteria persist in a non-replicating form (Young *et al.* 2009; Ahmad 2010; Lin and Flynn 2010; Patel *et al.* 2011; Ehlers and Schaible 2012; Ernst 2012). Latent tuberculosis infection is further characterized by the formation of granulomas which contain the harmful pathogen (Young *et al.* 2009; Ahmad 2010; Lin and Flynn 2010; Patel *et al.* 2011; Ehlers and Schaible 2012; Ernst 2012; Ramakrishnan 2012; Silva Miranda *et al.* 2012).

An important immune cell responsible for maintaining the granuloma structure as well as in the eradication of mycobacteria is the activated macrophage (Gideon and Flynn 2011; Kleinnijenhuis *et al.* 2011; Welin 2011; Ehlers and Schaible 2012). Activation of macrophages occurs classically through the activities of IFNγ and TNFα in response to Th1 T cell signals and results in the production of pro-inflammatory cytokines including IL-12 and TNFα. In addition, macrophages can be activated alternatively by IL-4 and IL-13 which is released from Th2 T cells and are characterised by the production of anti-inflammatory cytokines such as IL-10 and TGF β (Kleinnijenhuis *et al.* 2011; Welin 2011). The microarray analysis showed that IFNγ and TNFα, as well as their associated genes (interferon regulatory factor 10, TNFAIP2, TNFAIP3-interacting protein 1 (TNIP1), Fas ligand (TNF superfamily, member 6) and TNFRSF9) were significantly up-regulated in the long-term infected fish indicating a pro-inflammatory immune response and classical macrophage activation. This agrees with transcriptome profiles in mice infected with a low dose of *M. tuberculosis* H37Rv which displayed a shift towards Th1 T cell-mediated immune response and an increased expression of IFNγ and TNFα over the course of infection, in the transition from acute towards chronic disease (Gonzalez-Juarrero *et al.* 2009). Further pro-inflammatory pathway genes like CARD9 and positive regulators of inflammation such as DUSP2 and DUSP5 were up-regulated supporting the existence of an inflammatory response.

Besides macrophage activation, these cytokines play an important role in maintaining the granuloma structure, which is a necessity for containing and restricting the bacterium and controlling the disease (Ehlers and Schaible 2012; Ernst 2012; Ramakrishnan 2012; Reece and Kaufmann 2012; Torrado and Cooper 2013). In macaques infected with a low dose of *M. tuberculosis* an increase of IFNγ was detected at 6 weeks post-infection, which was interpreted as a transitional step in the control of the disease and establishment of the latent infection (Lin and Flynn 2010). In one zebrafish model for latent mycobacterial infection, an up-regulation in IFNγ

expression was detectable at 7 weeks post-infection alongside induction of NOS2b (Parikka *et al.* 2012). This phenomenon was observed in the present study as well, confirming the potential latent status of the infected zebrafish and the classical activation of macrophages. NOS2b is the zebrafish equivalent of the mammalian NOS2 or inducible oxide synthase (iNOS) (López-Muñoz *et al.* 2009; Parikka *et al.* 2012). In mice, iNOS is a marker for classical activated macrophages and is required for the control of *M. tuberculosis* infections (Flynn *et al.* 2011; Welin 2011). Induction of iNOS is triggered by IFNγ and followed by the production of nitric oxide, which is subsequently converted to reactive nitrogen intermediates (RNI). Reactive nitrogen and oxygen intermediates increase the antimicrobial activity of the macrophage and the capacity for killing mycobacteria (Co *et al.* 2004; Flynn *et al.* 2011; Welin 2011; Parikka *et al.* 2012; Torrado and Cooper 2013).

The up-regulation of NCF4 and IL-8 could be an indication of respiratory burst and the production of reactive oxygen intermediates (ROI). Neutrophil cytosol factor 4 (NCF4) regulates the phagocyte NADPH-oxidase (NOX2) which produces ROI from superoxide (Welin, 2011) and besides its chemotactic function of attracting inflammatory cells such as neutrophils, granulocytes, monocytes and T cells, IL-8 also induces respiratory burst (Ameixa and Friedland 2002; Wu *et al.* 2007; Shaler *et al.* 2012). While the role of neutrophils in established tuberculosis is still controversial and un-resolved (Korbel *et al.* 2008; Nandi and Behar 2011; Lowe *et al.* 2012), the importance of monocytes / macrophages and T-cells in disease refinement and latency is well established (Flynn *et al.* 2011; Gideon and Flynn 2011; Kleinnijenhuis *et al.* 2011; Ehlers and Schaible 2012). The production of IL-8 might support granuloma maintenance by attracting the participating cell types to replace infected or dead ones. Furthermore, IL-8 has been found to be highly expressed in latently infected humans with pulmonary tuberculosis and its expression profile has been proposed as a possible biomarker for latent infection in combination with FOXP3 and IL-12β gene expression (Wu *et al.* 2007; Walzl *et al.* 2011).

Looking at IL-8 and its chemotactic function for T cells, the up-regulation of another T cell attracting chemokine, CXCL 11, is notable. CXCL 11 is induced by IFNγ and IFN β and attracts activated T cells (Müller *et al.* 2010). Further, CXCL 11 has also been implicated as a possible biomarker in latent tuberculosis infections. In humans latently infected with pulmonary tuberculosis or tuberculous pleurisy, the expression levels of CXCL 11 were significantly higher than in healthy individuals or in patients with active disease (Thuong *et al.* 2008; Yu *et al.* 2012).

A number of antigen-processing and presentation and T cell receptor pathway genes were up-regulated including those associated with MHC class I (Protein kinase C, theta, RAS homolog gene families, α-2-macroglubolin receptor, Calreticulin and Beta-2-microglobulin) indicative of the activation of the adaptive immune response and indicating that besides the Th1-response, production of cytotoxic T lymphocytes (CD8+ CTLs) has been activated (Basu *et al.* 2001; Neefjes *et al.* 2011; Behar 2013; Green *et al.* 2013). Activation of CD8+ CTL cells, which have the ability to kill *M. tuberculosis*-infected cells, is mediated by Th1 cells and presentation of MHC class I-associated peptides including mycobacterial antigens in the cytosol (Andersen 1997; Ahmad 2010; Silva Miranda *et al.* 2012; Behar 2013; Green *et al.* 2013). Besides the knowledge of CD8+ in granulomas, the importance of these cells and their contribution to IFNγ production in *M. tuberculosis* infection has been long overlooked. Studies in mice and non-human primates have shown the relevance of these cells in *M. tuberculosis* infection (van Pinxteren *et al.* 2000; Chen *et al.* 2009; Behar 2013; Green *et al.* 2013). Van Pinxteren *et al.* (2000) showed the importance of CD8+ CTLs in latent *M. tuberculosis* infection in mice by finding that depletion of CD8+T cells, but not CD4+, was leading to an increased bacterial burden. In adult zebrafish challenged either with the acute *M. marinum* strain Mma20 or the chronic strain E11, no response of the MHC class I related molecules could be detected besides an up-regulating response at 1 d.p.i in the acutely infected fish (van der Sar *et al.* 2009). These fish had only been challenged for 6 days, while the chronically infected fish were sampled after

6-8 weeks which was probably not long enough to establish a latent infection, and therefore the immune response might not have turned towards an MHC class I mediated response. As described in Chapter 3 of this thesis, natural re-activation of the zebrafish mycobacteria was found after 2 m.p.i. The fish used in the experiment performed by van der Sar *et al.* (2009) were sacrificed before this crucial time point and therefore it is difficult to compare their transcriptional analysis results to the results described in the current study.

Another feature of CD8+ is the induction of apoptotic cell death in infected macrophages which is mediated via FASL and is TNF receptor dependent (Behar 2013; Divangahi *et al.* 2013; Ramakrishnan 2013)**.** FASL was up-regulated in this current study in addition to neuronal calcium sensor 1a (NCS1a), which suppresses necrosis. Directing cell death towards apoptosis assures that the phagocytosed bacteria remain within the dead cell which is the host's solution to containing bacterial dissemination (Behar 2013).

Although granulomas are important for the containment of mycobacteria, this persistent inflammatory response with its production of reactive intermediates causes tissue damage and oxidative stress (Saunders and Britton 2007). Therefore, regulation of the process is essential. Besides a chemotactic function for monocytes, polymorphonuclear leukocytes, and T lymphocytes, thioredoxin is known as antioxidant, which scavenges free radicals and helps to control the damage caused by antimicrobial responses (Circu and Aw 2010; Holmgren and Lu 2010). Thioredoxin is also implicated in the survival of *M. tuberculosis* during latency in mice (Hu and Coates 2009; Voskuil *et al.* 2011). Furthermore, other innate immune response genes were either down-regulated such as complement, CXCL12a and acute phase proteins or members of anti-inflammatory pathways were up-regulated such as cathepsin, which is important for generating peptides for the MHC class II pathway. Mice lacking cathepsin showed defects in class-II associated antigen presentation to control the damage from the persistent Th1-response (Riese *et al.* 1998; Shi *et al.* 1999). Down-

regulation of genes belonging to B-cell receptor pathways confirms this hypothesis, suggesting that MHC class II dependent pathways might be held off to regulate inflammatory and granulomatous responses.

In summary, the transcriptomic profile of long-term infected zebrafish displays all the features of a latent / sub-clinical disease state in which the bacteria are in a quiescent state within the granuloma, characterised by an established Th1 response including IFNγ and TNFα expression, IL-8 expression and activating and regulatory T cell responses including involvement of CTLs (Wu *et al.* 2007; Gonzalez-Juarrero *et al.* 2009; Walzl *et al.* 2011; Behar 2013).

5.1.2 Zebrafish with reactivated disease

Reactivation normally occurs when there is a change in the components of the immune system which serve to contain the pathogen. These changes can be induced by a variety of causes including age, other infections such as HIV co-infections or, as is the case here, stress (Lin and Flynn 2010; Gideon and Flynn 2011; Russell 2011). The latently infected fish were exposed to a number of stressors: higher stocking density, starvation and decreased ambient temperature (Magnadóttir 2006; Ramsay *et al.* 2006; Lawrence 2007; Ramsay *et al.* 2009b; Tort 2011). Ramsay *et al.* (2006) proved that, to obtain a stress response in the form of elevated cortisol levels from zebrafish kept at high stocking density, starvation was essential. In addition, it was decided to decrease the water temperature to slow down the metabolism of the fish, this having a negative effect on the immune response (Le Morvan *et al.* 1998; Magnadóttir 2006; Uribe *et al.* 2011). Following removal of the stressors, it is hypothesised that the mycobacteria can regain function faster than the fish immune system and thereby use that window to break dormancy and induce re-activation of the disease.

The fish were given a couple of days for recovery after treatment. Recovery from higher stocking density alongside starvation takes less than 24h (Ramsay *et al.*

2006; 2009a). Transcriptomic analysis for stressed x infected fish might be expected to provide evidence for the re-activation of the disease. In general, a broader spectrum of immune related genes were differentially expressed in stressed x infected fish than in infected fish including induction of CCL 8, IL-1β, IL-15ra, SOCS1, NFKBIAA, NFKBIAB, IRAK3, MMP9 and IRF1 as well as IL-8, TNFα, NOS2b and CXCL 11. Other genes appeared to be depressed, such as TNIP1 and TNFAIP2, in addition to CXCL-C1c, CXCL-14, TGF β, CCL-C20c and NFKB2.

Induction of MMP9, CXCL-C1c, IRAK3, IL-8 and IL-1β has been associated with innate immune response to *Salmonella* infection in zebrafish embryos (Stockhammer *et al.* 2009) and in connection with TNF, INF γ and IL-1β expression during *M. marinum* infections in zebrafish (van der Sar *et al.* 2009). Changes in gene expression of TNFα, IL-1β and IL-8 has been shown previously in connection with zebrafish immune responses to *Edwardsiella tarda* infection (Pressley *et al.* 2005) and *M. marinum* (Clay *et al.* 2008). In addition, MMP9 has been shown to play an important role in early granuloma formation in response to mycobacteria, as it is induced by the bacterium in the host to recruit monocytes and macrophages to the site of infection (Volkman *et al.* 2010; Salgame 2011; Ramakrishnan 2012; Reece and Kaufmann 2012). In mycobacterial infections, MMP9 has been implicated in the activation IL-1β and thereby promotion of the inflammatory response (Sheen *et al.* 2009; van der Sar *et al.* 2009). It has also been found that a high-level of MMP9 has been found in active tuberculous pleurisy in humans, correlating with caseous necrosis in the granuloma (Sheen *et al.* 2009; Salgame 2011).

In humans, reactivation of tuberculosis is caused by the depletion of one or more of the essential immune components, these including TNFα, IFNγ and Th1 cells (Saunders and Britton, 2007), which can result in incomplete granuloma formation or errors in the maintenance of pre-existing ones (Saunders and Britton 2007; Shaler *et al.* 2012; Ramakrishnan 2013).

Transcriptomic analysis of the infected x stressed fish appears to demonstrate the occurrence of all these phenomena. No IFNγ was detectable and the expression levels of TNFα and NOS2b were much lower than for the latent infected fish. For instance, compared to the fish with presumed latent infection, hardly any T cell receptor-related genes were induced and other genes such as B2M, TNFSF13b or TAPASIN were suppressed. It has been shown in humans, mice and fish that stress-induced elevation of corticosteroids has a suppressing effect on T and B cell immunity (Harris and Bird 2000; Magnadóttir 2010; Hunzeker *et al.* 2011). In comparison to the stressed x uninfected fish, the fold change of the stress x infected fish was always lower in these genes suggesting that the effect of stress alone was not as strong as the effect of stress x infection. This lack of a T cell response could explain the lack of IFNγ that was observed in this study.

Depletion of IFNγ and TNFα appeared to resulted in a lower number of activated macrophages (or level of activation), indicated by the lower level of NOS2b expression. Mice deficient in either of these cytokines have been shown to be highly susceptible to *M. tuberculosis* infection alongside poor development of granulomas (Ehlers and Schaible 2012; Shaler *et al.* 2012; Ramakrishnan 2013; Shaler *et al.* 2013; Torrado and Cooper 2013). A lack of NOS2b can also be associated with necrosis. It has been shown in mice and other animal models that deficiency in NOS2 or Th1-response can result in the presence of necrotising granulomas (Ehlers and Schaible 2012). Necrosis in the centre of the granuloma has been shown to correlate with the transition of latent to active tuberculosis (Dannenberg Jr and Sugimoto 1976; Russell *et al.* 2009; Kim *et al.* 2010). The most recent theory on the establishment of the caseum in the granuloma centre proposes that immune cells such as macrophages, especially foam cells, and neutrophils in the centre, undergo *Mycobacterium*-induced necrosis (Divangahi *et al.* 2010; Ehlers and Schaible 2012). The dying cells release their content into the granuloma centre, which leads to the accumulation of debris and lipids (Divangahi *et al.* 2010; Kim *et al.* 2010; Ehlers and Schaible 2012; Shaler *et al.*

2013). The caseating centre promotes bacterial replication as mycobacteria have been proven to metabolise the lipids, it also protects them to a certain degree by walling them off from the host immune response (Ramakrishnan 2013; Shaler *et al.* 2013). Increasing caseation and bacterial replication leads to liquefaction of the centre, which ruptures and then the bacteria are released causing reactivation (Kim *et al.* 2010; Shaler *et al.* 2013).

Reactivation of *M. marinum* in the challenged zebrafish in this study is further supported by the suppression of the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-kB) complex. The results obtained showed a down-regulation of nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) also known as NFKB2 and the induction of NFKBIAA and NFKBIAB, both zebrafish homologs of the human nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFKBIA), which inhibit the NF-kB complex. Inhibition of TNF-mediated NF-kB signalling pathway has been found to promote *M. tuberculosis* infections (Fallahi-Sichani *et al.* 2013). The authors showed that inhibition of the NF-kB signalling led to uncontrolled growth of the bacteria within the granuloma, while an over-expression of the same pathway caused excessive inflammation and the regulation of NF-kB signalling is a crucial element in the control of mycobacterial infection.

Glucocorticoids have been shown to have an inhibitory effect on the NF-kB signalling pathway by inducing the expression of NFKBIA. Furthermore, the connection of ROS and activation of the NF-kB signalling have also been implicated, although the underlying mechanisms are still unknown (Tripathi and Aggarwal 2006). NF-kB signalling is also suggested to inhibit apoptosis (Baichwal and Baeuerle 1997; Barkett and Gilmore 1999; Tripathi and Aggarwal 2006). The increased number of differentially induced cell death associated genes seems to confirm the re-activation theory and the connection with NF-kB signalling.

Another explanation for the increased number of cell-death related genes has been suggested for zebrafish embryos as well as in human and murine cell cultures infected with *M. marinum* or *M. tuberculosis*. The infected macrophages in these studies displayed multiple cell death caused by the ESAT-6 protein, which is secreted from RD1-locus of the pathogenic bacteria (Davis and Ramakrishnan 2009; Volkman *et al.* 2010; Ramakrishnan 2012). These findings support another recent theory of *Mycobacterium*-induced caseum formation in the granuloma centre. The RD1 locus is associated not only with the death of infected macrophages, but also relates to induction of aggregation and recruitment of macrophages, which aids granuloma formation at the early stage of infection and might contribute to necrosis and caseum development in the centre of chronic granulomas (Davis and Ramakrishnan 2009; Volkman *et al.* 2010; Ramakrishnan 2012).

Taken together, the transcriptomic profile of the infected and stressed zebrafish is indicative of an inflammatory response, this being highlighted by IL-1β, TNFα, IL-8 and MMP9 induction, in addition to a hardly detectable induced adaptive response. Due to the lack of IFNγ and reduction of TNFα and NOS2b expression, it appears that the latent disease might have been reactivated, possibly as a result of the failure of the key mechanisms involved in maintaining granuloma integrity.

Future investigations have to be performed to support the findings made in this current study. As described before, commercial Oligo microarrays are designed to give a profiling analysis of the complete transcriptome. Meijer *et al.* (2005) compared three commercially available zebrafish microarrays from Sigma, MWG and Affymetrics to investigate the transcriptome profiling of adult zebrafish at the late stage of chronic tuberculosis caused by *M. marinum* infection and discovered the lack of important immune related genes on these arrays including IL-1β, IL-12, iNOS, TLRs and TNF. Customised microarrays on the other hand can be designed to fit the experimental design and regarding the current study, a customised array designed only with

immune and cell death related genes might have given further insights into the immune response of adult zebrafish to *M. marinum* infections.

Unfortunately, due to the occurrence of re-infection during the experiment as described in Chapter 3, no fish were remaining after the microarray analysis to perform additional histological analysis of the infected and stress x infected fish to confirm their disease stages by granuloma and *M. marinum* distribution.

5.2 Conclusions

In this study, a latent infection of *M. marinum* in a population of adult zebrafish was established over a period of 6 months. The latent infection displayed similar features to those described for human and non-human primate models, including the absence of clinical signs and the expression of particular genes thought to be correlated with latent infections. The reactivation of the latent disease as a result of exposure to key stressors has been demonstrated in the zebrafish model, this being one of the proposed routes for the reactivation of tuberculosis in humans. The transcriptomic profile for fish subject to both stress and infection displayed expression of active tuberculosis-related genes.

From the results of the present study, it is suggested that it is possible to mimic both latent and reactivated tuberculosis infections in adult (*i.e*. fully immuno-competent) zebrafish, using *M. marinum* as the infecting pathogen and that an appropriate infective state may be achieved over a period of 6 months. Such a model can provide an important new tool for gaining understanding of the mechanisms involved in *M. tuberculosis* infection and granuloma formation in humans.

6 General discussion

6.1 *General discussion*

Tuberculosis is a global epidemic disease, which still causes major disease problems in humans. For decades, it was thought to be beaten due to the discovery of the causative pathogen, *M. tuberculosis*, the effectiveness of antibiotics and the development of the BCG vaccine. This optimism was tempered by its resurgence in the 1980s, occasioned by the development of multidrug-resistant tuberculosis strains and the link between tuberculosis and HIV. In 1993, the re-appearance of tuberculosis was declared as a global health emergency by the WHO (Zumla *et al.* 2013).

Since then, increasing numbers of multidrug- (MDR), excessive drug- (XDR) and totally drug-resistant (TDR) *M. tuberculosis* strains have been reported, which complicate and prolong the treatment of tuberculosis. The resistance of *M. tuberculosis* strains is a result of the use of old treatment drugs and inconsistency in the treatment completion. The drugs currently used in disease treatment were discovered over 50 years ago. Furthermore, the length of application time required for these drugs to cure the disease, in combination with their high price make it difficult for poor people or people living in countries entirely lacking or having minimal healthcare systems to complete the necessary treatment (Das *et al.* 2013; WHO 2013; Zumla *et al.* 2013). Unfortunately, the same group of people are highly susceptible to tuberculosis due to their compromised immune systems, creating a vicious circle in which tuberculosis persists (WHO 2013). The development and progression of *M. tuberculosis* is not only highly dependent on the individual host immune status, it is also reliant on the bacterial isolate which causes the infection, making the prediction of the disease outcome and treatment very difficult.

The complex pathogenesis of tuberculosis with its different disease stages, which tend to overlap, is still not completely understood, especially the latent disease stage,

which causes trouble in diagnosis since reliable distinguishing tests are not available. In addition, the niche where the bacteria are hiding during the latent disease stage has still not been completely identified which makes the research for new therapies and concentrated treatment more difficult (Gideon and Flynn 2011; Das *et al.* 2013).

Traditional model organisms, such as mice, are not appropriate models for tuberculosis and the use of non-human primates which mimic the disease in all its aspects is difficult to defend on ethical grounds. The zebrafish, with its natural *M. tuberculosis*-related pathogen *M. marinum*, has been placed under the spotlight in tuberculosis research, and has already proven valuable as a model for granuloma formation in tuberculosis (Ramakrishnan 2013).

Granuloma formation in zebrafish is well understood during the early stages of disease progression due to the comprehensive work carried out by the Latita Ramakrishnan Lab who have used the zebrafish embryo model infected with *M. marinum*. The transparency of the zebrafish embryo allows real-time monitoring and investigation of granuloma formation and the interaction of the contributing bacteria by fluorescent labelling of the latter. Using these advantages, the Ramakrishnan Lab has contributed some new insights into the understanding of granuloma formation and discovered some remarkable new ways by which the pathogen manipulates host macrophages (Cosma *et al.* 2008; Davis and Ramakrishnan 2009; Volkman *et al.* 2010; Ramakrishnan 2012).

All these discoveries have been made in zebrafish embryos or larvae, which lack an adaptive immune response because it has not yet developed (Trede *et al.* 2004; Lieschke and Currie 2007; White *et al.* 2008a; Novoa and Figueras 2012). Thus, only innate immune response is involved which makes the discoveries difficult to transfer to further advanced disease stages such as chronic and latent disease. In humans, the adaptive immune response is the key player in fighting advanced tuberculosis disease stages by restricting intracellular bacterial growth through macrophage activation and

reactive intermediate production and killing of infected cells by cytotoxic T cells (Flynn *et al.* 2011; Welin 2011; Parikka *et al.* 2012; Torrado and Cooper 2013).

Furthermore, adaptive immunity is important for completing the formation of granuloma structure, which separates the intracellular bacteria from the surrounding tissue (Basaraba 2008; Barry *et al.* 2009; Reece and Kaufmann 2012). The most recent understanding of the granuloma is based on the results from the Ramakrishnan Lab and it appears that the granuloma is a dynamic structure with cells being constantly replaced and other cells trafficking through it (Cosma *et al.* 2008; Davis and Ramakrishnan 2009; Volkman *et al.* 2010; Gideon and Flynn 2011; Ramakrishnan 2012; Ramakrishnan 2013).

Whether or not the same dynamic nature of the granuloma is detectable in early granuloma formation in adult zebrafish that exhibit a fully functional adaptive immune response has to be further investigated. Studies focusing on granuloma formation or the host response to *M. marinum* in the adult zebrafish are scarce and have mostly focused on the early stages of acute or chronic disease stages (Meijer *et al.* 2005; Swaim *et al.* 2006; van der Sar *et al.* 2009; Parikka *et al.* 2012). Further investigations and detailed information about the long-term immunopathogenesis of *M. marinum* infections in adult zebrafish are required to fill the gaps and extend the understanding of this disease.

In this thesis additional insights into the immune response of adult zebrafish to chronic *M. marinum* infections were gained, showing that granuloma formation and progression is similar to that found in humans and non-human primate models, as highlighted in Chapter 3. Granuloma formation started with an aggregation of macrophages, which further developed into non-caseating and caseating granulomas found in the same tissue 2 months post-infection. Agreeing with the human and non-human primate models, the bacterial burden was higher in the caseating granulomas than in the non-caseating ones, and the development of a fibrotic cuff around the caseating granulomas was observed. The only characteristic missing in the zebrafish

model was the development of calcified granulomas for which longer monitoring of granuloma formation in zebrafish would be necessary. Calcification in humans is the result of either healed granulomas or it occurs when the caseous granuloma centre liquefies, releasing their contents into the surrounding tissue after bursting. Altogether, the results of the current study support the use of the zebrafish and *M. marinum* as a model for granuloma formation in human tuberculosis infections beyond the embryo and larval stages of the zebrafish, *i.e*. in adult zebrafish.

Besides the importance of investigating disease progression *in vivo,* which has contributed a large part to our understanding and knowledge about host-pathogen interactions (Beignon *et al.* 2014), the development of zebrafish cell cultures to further investigate these host-pathogen interactions *in vitro* still lags behind the standards established for mammalian cultures. Preliminary work to develop a macrophage culture from individual zebrafish kidneys was performed in this thesis to investigate the interaction of *M. marinum* and zebrafish macrophages *in vitro*. Although the initial attempts looked promising, further optimisation clearly needs to be performed.

The ability to use the *in vivo* zebrafish-*M. marinum* model beyond the larval development stage as a tool for studying human tuberculosis infection, requires further understanding of the immune response of the model organism during infection in its adult life stage, starting from the initial infection to the establishment of long-term infection. This was investigated in Chapter 4 by focusing on the innate immune response in the kidney of adult zebrafish with chronic *M. marinum* infection over the first 14 days of infection. The results further confirmed the similarity of *M. marinum* infections in zebrafish to *M. tuberculosis* infections in humans and mice. As with *M. tuberculosis, M. marinum* appears to manipulate the host immune response. This was shown by the delayed IFNγ response which, was hardly observable before 14 days post-infection and by continued TNFα expression at 14 days-post infection in the infected fish investigated in this current study.

Similarly to *M. tuberculosis* infections in mammals, the outcome and severity of *M. marinum* infections in zebrafish, as well as other host organisms, is dependent on the mycobacterial isolate. While *M. marinum* NCIMB 1297 caused chronic granulomatous infections in the challenged zebrafish (Chapter 3+4), isolate NCIMB 1298 did not induce any considerable changes in the immune response of the fish. The disease outcome is strongly dependent on the virulence of a *M. marinum* isolate. The choice of isolate and determination of the infective dose administered to the model organism is an important consideration for future work. Depending on the pathogenicity of the *M. marinum* isolate employed, the final outcome of the disease can vary between acute and chronic disease, although an identical injection dose (cfu mL^{-1}) had been administered to the fish.

The delayed immune response following mycobacterial infection also coincided with the detection of granuloma formation, as shown in Chapter 3. Correlation of granuloma formation with the onset of the adaptive immune response has been described in other models (Lin and Flynn 2010; Ramakrishnan 2013). In the mouse model, the initiation of the T-cell response correlated with the occurrence of the granulomas observed histologically, and also coincided with a plateau of observable bacterial growth (Lazarevic *et al.* 2005; Lin and Flynn 2010). Similar observations were made in the non-human primate model (Lin *et al.* 2006; Lin *et al.* 2009c). This phenomenon reflects the close relationship between adaptive immune response, bacterial growth and granuloma formation, which is important in controlling *M. tuberculosis* infections in humans, non-human-primates and mice. Regarding the results obtained in Chapters 3 and 4 of this study, a similar relationship between the three factors can be assumed for *M. marinum* infections in adult zebrafish.

To fulfil the criteria for provision of an ideal model of human tuberculosis infections, the zebrafish-*M. marinum* model has to provide the ability to produce latent *Mycobacterium* infections, which can subsequently be reactivated. A previous study performed by Parikka *et al.* (2012) had already proven that the zebrafish- *M marinum*

model is capable of producing latent infection, and this group managed to break latency by exposing the fish to gamma radiation. Natural reactivation of the latent infection in humans is thought to be linked to immunosuppression of the host immune system. An attempt to mimic this natural reactivation in the adult zebrafish-*M. marinum* model was described in Chapter 5. Adult zebrafish infected long-term with *M. marinum* isolate NCIMB 1297 (a different isolate to that used by Parikka *et al.* (2012), were exposed to a number of environmental stressors including lowering ambient temperature and increased stocking density. The fish exposed to the stress factors displayed similar transcriptome profiles to those previously found in animal models and humans with reactivated *M. tuberculosis,* while the fish which were infected long-term but not exposed to stress displayed gene profiles similar to latent infections with *M. tuberculosis*.

This thesis has thus demonstrated that *M. marinum* can both manipulate the zebrafish host immune response and induce a latent infection, which can subsequently be reactivated in a similar way as has been observed in *M. tuberculosis* infections in human or the non-human primate models.

A general difficulty and time-consuming aspect of the work described in this thesis was the adaptation and optimisation of existing protocols and experimental techniques in the Institute of Aquaculture to a smaller scale, so they were applicable to the zebrafish. Furthermore, the dissection of individual organs from adult zebrafish and the subsequent extraction of RNA or DNA from these organs is not a common technique in the zebrafish model, where instead experiments have been carried out with embryo or larval developmental stages as summarised in Chapter 1.5.2. Investigations in adult zebrafish have previously used RNA and DNA extracted from the entire fish or by pooling a number of organs from several individuals to achieve higher RNA yield to execute molecular analyses (Meijer *et al.* 2005; Pressley *et al.* 2005; Lin *et al.* 2007; Rodriguez *et al.* 2008). Especially in the investigation of tuberculosis, where every parameter in the surrounding environment and the individual

host health status affects the outcome of the disease (active, latent or reactivation), the analysis of individual fish is important while pooling samples increases the risk of obscuring potentially important aspects of the disease. The results presented in this thesis show substantial variations in the individual response of adult zebrafish to the pathogenic *M. marinum* isolate NCIMB 1297, as shown by the large standard errors observed in Chapters 3 and 4. In particular, the gene expression results of TNFα, IL-12 and IFNγ at 14 d.p.i., showed that the fish investigated were at different stages of the immune response, with some fish already having activated the adaptive immune response, detectable by the strong induction of IFNγ expression, while the remaining fish displayed low or negligible IFNγ expression. Interferon gamma expression not only provided evidence for the onset of the adaptive immune response as described in Chapter 4, but also aided distinction of the latent disease stage from the reactivated one in Chapter 5 and can thus play an important role in disease diagnosis.

The results presented in this thesis not only support the usefulness of the adult zebrafish-*M. marinum* model for studying human latent human tuberculosis infection, but also allow the transfer of knowledge relating to *M. tuberculosis* infection in humans to the study of *M. marinum* infection in fish. This work raises questions and concerns about how *M. marinum* infections in farmed fish are treated and about the assumption that after treatment with antibiotics the disease is cured when there is a high possibility that *M. marinum*, similar to *M. tuberculosis*, survives in a latent disease state and has the ability to hide within the host, until the opportunity for re-activation of the disease occurs.

The zoonotic nature of *M. marinum* makes contact with this pathogen not only a risk for fish populations, but it is also a threat for people coming into contact with infected fish such as farm workers, fishmongers or people who maintain a home aquarium (Decostere *et al.* 2004; Hastein *et al.* 2006; Gauthier and Rhodes 2009; Jacobs *et al.* 2009; Francis-Floyd 2011; Lewis and Chinabut 2011). Recently, it has been shown that *Mycobacterium avium* in fish products can survive smoking and frying

and might be also a potential risk for consumers of fish products because this *Mycobacterium* strain can also be transmitted from fish to humans (Klanicova *et al.* 2013). Furthermore the findings of Klanicova *et al.* (2013) raise the question of whether other *Mycobacterium* species such as *M. marinum* and *M. salmoniphilum* survive the smoking process and whether ingestion of *Mycobacterium spp*. contaminated food is a potential risk for human health.

Although it has been reported that the zoonotic risk of infection is considerably higher through wounds on the hands or feet, than through consumption of fish products, it is a possible route of infection which has to be considered.

Therefore the identification of suitable biomarkers is not only urgently needed for the treatment and detection of human tuberculosis, there is also a requirement for such tools in aquaculture.

6.2 *Future work*

Although the results presented in this thesis did not demonstrate a direct effect of the injection methods on the zebrafish's immune response, further investigation needs to be performed to verify this, especially regarding the local immune response at the injection site. Considering the small size of the fish and the needle size used, smaller equipment such as a Hamilton syringe might be an alternative. These syringes also allow the injection of smaller doses then applied in this study which would further reduce the stress on the zebrafish's abdomen.

In addition to the organ highlighted in this study, the kidney, the immune response of adult zebrafish to *M. marinum* should be investigated in other organs e.g. spleen and liver to complete our understanding of the immunopathogenesis of this disease.

In future, gene expression data has to be compared to protein levels to investigate how much of the transcribed mRNA is translated into proteins. The correlation between mRNA and protein levels can be affected by a number of

processes such as translation, protein stability or protein modification. Depending on the system, the correlation can be as low as 40% (Vogel and Marcotte 2012). Therefore, the additional investigation of the expressed immune cytokines by functional assays is important, not only to provide estimates of actual protein levels but also to get an idea of the complexity of host manipulation by mycobacteria.

This study has provided an insight into the innate immune response over the first 14 days post infection as well as the state of the immune response through transcriptional profiling at 6 m.p.i., but the gap between these two studies has to be filled by further investigations. In addition, granuloma development has to be observed after the 2 m.p.i. time point which might also give insights regarding the sudden re-activation of the disease, as described in Chapter 3 of this thesis.

The Casper zebrafish, a transparent mutant, in combination with the use of fluorescently labelled bacteria, provides the tools to monitor granuloma formation and disease progression in real time in the adult fish over a long period. In addition, the use of fluorescent bacteria would allow the exact localisation of the granuloma in the zebrafish and the use of laser dissection microscopy would enable the dissection of the granuloma for further analyses, e.g. gene expression. Gene expression from isolated granulomas from humans is only possible when removing them during biopsy, surgery or autopsy which normally happens during the active disease stage (Fenhalls *et al.* 2002; Zhu *et al.* 2003) because the precise tools for the detection of latent disease stages in humans are still missing. For further understanding the latent disease stage and determining which populations of mycobacteria in the host are actually responsible for the reactivation of the disease, new mutants of the *Mycobacterium* might be of assistance. It is known that specific genes *of M. tuberculosis* are being up-regulated during dormancy such as DosR and DosS (Lin and Ottenhoff 2008; Hegde *et al.* 2012). By labelling *M. marinum* or *M. tuberculosis* by insertion of a fluorescent marker gene into the genome, which would be expressed

when the latency-related genes are switched on, the determination of the time point at which bacterial dormancy occurs and the location in the host may be possible.

In regard to aquaculture, the development of non-lethal detection methods for *Mycobacterium spp*. in infected fish stocks, such as blood or mucus analysis, are desirable to minimise the potential risk for farm workers, fish handlers and consumers as commercial fish species such as salmon, cod and trout are known to be carriers of zoonotic mycobacteria. Furthermore, these detection methods are also needed for private home aquarists and pet stores where zebrafish and goldfish are a potential risk for humans.

The zebrafish meets all the requirements for a good model for mycobacterial infections but the techniques are still not standardised enough and are mostly adapted from mammalian research, i.e. route of infection and housekeeping genes. Additional research is required in the zebrafish model because determination of a large number of important pathways and immune cells are still lacking. It is assumed that, due to the existence of similar sequences and connections, the pathways and immune cells in fish behave in the same fashion as their homologues in humans, however, fish do have some additional components and similarly lack other important ones.

The majority of drugs or treatment methods discovered in previous animal models for tuberculosis fail nowadays when transferred from the model organism to humans. Although it is clear that the concept of the perfect model organism for human tuberculosis remains a dream, the adult zebrafish model may provide opportunities to assist the development of treatments that are applicable for humans.

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